Bacteriophage as a biocontrol tool for foodborne pathogens

A thesis submitted in partial fulfilment of the requirements for the Degree of Master of Science in Microbiology at the University of Canterbury

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Abstract

Despite the widespread use of interventions such as chemical sanitizers, UV light, heat (pasteurisation) and high pressure to improve food safety, incidences of foodborne disease continue to increase worldwide, prompting investigation into alternative controls. One such alternative is the use of bacteriophage (phages) for biocontrol of foodborne pathogens. As part of this research three *E. coli* phages and two *Listeria* phages were isolated, characterised and applied to investigate their biocontrol potential. Phages were first characterised by host range analysis, transmission electron microscopy and adsorption assays. These results suggested that two *E.coli* phages are members of the *Myoviridae* family, and the other is a member of the *Podoviridae* family, all three phages have a limited host range and are fast adsorbing. Whereas, the *Listeria* phage CTLLm3 is a member of the *Siphoviridae* family, has a broad host range and is fast adsorbing. As the *E. coli* phage AAPEc6 and the *Listeria* phage CTLLm3 showed biocontrol potential they were further investigated through kinetic and applied experiments on meats and biofilms respectively. The results of the kinetic experiments suggested that AAPEc6 was stable from pH 3-7 and a temperature range of -20°C to 50°C, similarly, CTLLm3 was stable from pH 5-7 and at a temperature range of -20°C to 40°C. Both phages showed optimal host lysis between 30°C and 40°C and at MOI values of 10 and 100. The application of AAPEc6 onto meat samples contaminated with *E. coli* resulted in a significant reduction in bacterial numbers, meanwhile the application of CTLLm3 onto *Listeria* biofilms significantly reduced biofilm mass. The results of these experiments will contribute towards our understanding of phage application as a biocontrol tool against foodborne pathogens.

Chapter 1: Introduction

1.1. General Introduction

Foodborne diseases consist of a group of illnesses caused by exposure to a range of different pathogens through contaminated food or water (Rocourt, Moy et al. 2003). Each year across the world, food related illnesses cause a significant number of infections, in 2010 alone an estimated 600 million cases of illness worldwide were attributed to foodborne hazards (Vos, Flaxman et al. 2013). These illnesses range in severity from mild to those that result in death (Newell, Koopmans et al. 2010). Although foodborne illnesses have historically been local and contained, they are increasingly emerging as widespread, or global outbreaks which affect a significant number of individuals (Hall, Vally et al. 2008). Foodborne diseases can infect any individual however, pregnant women, children, the elderly and immune-compromised individuals are at a higher risk than others (Ramaswamy, Cresence et al. 2007). Due to the serious consequences associated with foodborne infection and the potential for subsequent outbreaks, interventions such as the use of ultra violet light (UV) irradiation, pasteurisation, high pressure and chemical sanitizers have been implemented (Garcia, Martinez et al. 2008). Despite these efforts, incidences of foodborne disease continue to increase worldwide each year (Hagens and Loessner 2007). This increase is attributed to factors including changes in human behaviour, industry, technology and globalization, which have contributed to the evolution and rapid spread of pathogens and consequently infections across the world (Altekruse, Cohen et al. 1997). Therefore alternative methods of pathogen control are required, one such alternative is the use of bacteriophages as a biocontrol tool (Hagens and Loessner 2007).

1.2. Foodborne Disease

Foodborne diseases develop following consumption of contaminated food or water after an incubation period of between one to seven days (Rocourt, Moy et al. 2003). Despite the serious nature of these illnesses, a significant number of cases are undiagnosed or unreported, due to the nature of the illnesses and limitations in diagnostic capabilities, especially in the developing world (Tauxe 1997).

Contamination of food and related products can occur at any stage during preparation or processing, from the initial farm environment to final stages of preparation, resulting in illness or outbreaks (Newell, Koopmans et al. 2010). While the majority of reported cases are from meats, seafood, poultry and eggs a number of cases arise from the domestic kitchen environment (Meredith, Lewis et al. 2001). Although the most common cause of contamination occurs following contact with unclean surfaces there are a number of other factors to be considered (Nyachuba 2010). Firstly, the pooling of raw material can result in the contamination of other products or the final product due to contact with a single contaminated component (Nyachuba 2010). Secondly, food and related products are generally handled by more than one person and are processed through a range of different equipment (Nyachuba 2010). Thirdly, in continuous food production systems the presence of food, water and adequate temperature may contribute to the growth and maintenance of microbial populations (Nyachuba 2010). As well as this, failure to continuously monitor and maintain a clean environment can contribute to the formation of biofilms (Donlan 2002). Biofilm formation is a significant threat as once establishment has occurred biofilms are almost impossible to remove as they are highly resistant to cleaning and sanitation procedures. Further to this cleaning a well-established biofilm may result in further contamination as daughter cells are dispersed into the surrounding environment (Kumar and Anand 1998).

The changing nature of foodborne illnesses around the world has been attributed to several factors including, the large scale production and widespread distribution of food, increasing globalisation, eating outside of the home environment, changing environments, emergence of new pathogens and an increasing proportion of at-risk consumers (Altekruse, Cohen et al. 1997, Nyachuba 2010). These factors combined have resulted in the more frequent movement of foods and pathogens across the world, increasing the potential for global outbreaks (Newell, Koopmans et al. 2010). As human factors have changed over time pathogens have adapted, evolved and remained successful (Newell, Koopmans et al. 2010).

1.2.1 Foodborne disease in New Zealand

The serious nature of foodborne diseases has prompted much research to investigate the burden and prevalence associated with these illnesses. A study conducted in 2010 by Lake *et al* considered the application of the disability-adjusted life years (DALYs) approach and cost of illness (COI) approach to estimate the burden of disease for six potential foodborne illnesses in New Zealand. The application of both approaches resulted in similar rankings

with perinatal and nonperinatal *Listeria* ranked third and fourth respectively, and *E. coli* ranked fifth. These rankings highlight the burden associated with foodborne illness in New Zealand (Lake, Cressey et al. 2010).

In 2011 a study conducted by Cressey & Lake (2011) applied the overseas Scallan model with multipliers to estimate the number of cases of illness, hospitalizations and deaths occurring due to food related illness. The study estimated that approximately 17 fatalities will occur each year as a result of the 24 pathogens that are transmissible through food. 65% of these fatalities will be caused by bacteria; the remaining percentage will be equally divided between viruses and parasites (Cressey and Lake 2011). Although this study provides useful insight into foodborne infection, the output of the study is considered hypothetical as the applicability of overseas models to New Zealand models is unknown (Cressey and Lake 2011).

Another New Zealand study by Lake et al (2000) investigated the number of annual infectious intestinal diseases caused by foodborne pathogens in New Zealand. Data gathered from hospital and ministry of health records indicated that the estimated number of cases per year was 119,000. This included 119,000 visits to general practitioners, 400 hospital admissions, 22 cases of long term illness and 2 deaths. All 119,000 cases were thought to be related to foodborne pathogens, however, the total number of cases potentially related to foodborne disease is estimated to be around 832,000. The number of days lost in terms of production and leisure time was estimated at 497,000. The study concluded that foodborne disease in New Zealand represents a major public health concern, and the number of reported cases is increasing, highlighting the need for alternative methods of pathogen control (Lake, Baker et al. 2000).

1.2.2 Emergence and transmission of foodborne pathogens

Pathogen control has advanced over the years and procedures have been successfully implemented to reduce pathogen spread (Tauxe 1997). However, in recent times there has been an increase in emerging pathogens as well as established pathogens (Altekruse, Cohen et al. 1997). As new pathogens emerge, established pathogens adapt and evolve new characteristics allowing them to continue infecting, the presence of these pathogens in the food chain poses a serious threat (Tauxe, Doyle et al. 2010). Research into this increasing trend has highlighted a number of contributing factors including globalization which drives changes in consumer needs, and an increase in the proportion of immuno compromised

people and carriers (Tauxe, Doyle et al. 2010). Carriers are generally human or animal hosts that harbour concentrations of the pathogen within them, however they are asymptomatic (Tauxe 1997). Consumption of and interaction with such carriers can result in exposure to the pathogen and thus illness (Tauxe, Doyle et al. 2010). These emerging zoonotic pathogens can spread globally, show resistance to some antibiotic and chemical treatments and can withstand general food preparation techniques that have previously been successful in preventing pathogen spread (Tauxe 1997). Such pathogens are a significant public health concern as they remain in foods that look, smell and taste normal (Tauxe 1997).

1.3 Foodborne Pathogens

There are a range of foodborne diseases caused by over 200 known pathogens, including bacteria, viruses, parasites, fungi and in some cases toxins and chemicals (Taege 2004). The consumption of these pathogens can result in the development of a range of symptoms and illnesses (Rocourt, Moy et al. 2003). The most commonly identified pathogens include *Campylobacter* spp, *Salmonella* spp, *Shigella* spp., and *Escherichia coli* O157:H7. Although *Listeria monocytogenes* is not commonly identified, it has a high fatality rate, making it a pathogen of concern (Taege 2004). A summary of common foodborne pathogens, at risk food types, and clinical symptoms manifested in patients are provided in Table 1.1.

Table 1.1: Summary of common foodborne disease types (Parashar, Quiroz et al. 2001, Acheson and Fiore 2004, Taege 2004, Mathusa, Chen et al. 2010)

Summary of common foodborne diseases		
Pathogen	**Foods associated with illness**	**Symptoms**
Bacteria		
***Salmonella* spp.**	Eggs, poultry, fruits, vegetables, meats, ice cream	Fever, diarrhoea (occasionally bloody), abdominal cramps
Camplylobacter jejuni	Chicken, unpasteurized milk, dairy products, water	Fever, diarrhoea (occasionally bloody)
***Shigella* spp.**	Fruits, vegetables and shellfish	Can produce invasive dysentery syndrome, fever and bloody diarrhoea.

Listeria monocytogenes	Deli meats, soft cheeses, unpasteurized milk, dairy products, vegetables, ready to eat foods.	Symptoms differ depending on the host. General symptoms are flu-like, however may develop into conditions such as meningitis and febrile gastroenteritis
***Vibrio* spp.**	Shellfish	Illness tends to be seasonal. Symptoms include watery diarrhoea, abdominal cramps, nausea, vomiting and fever.
***Yersinia* spp.**	Raw pork, unpasteurized milk, dairy products, water	Fever, abdominal pain, bloody diarrhoea.
Escherichia coli O157:H7	Hamburger, salami, alfalfa sprouts, unpasteurized milk, dairy product, lamb ground beef, vegetables, fruits.	Abdominal cramps, bloody diarrhoea, little or no fever. Can result in the development of fatal conditions such as acute or chronic renal failure.
Viruses		
Norwalk	Shellfish, ready to eat foods, water, food handlers.	Nausea, vomiting, abdominal cramps, diarrhoea, headache, fever.
Hepatitis A	Shellfish, infected food handlers, fresh produce contaminated during harvesting or distribution.	Fever, anorexia, nausea, vomiting, diarrhoea, malaise, myalgia.

1.3.1 Listeria

L. monocytogenes is the prokaryote responsible for causing listeriosis in animals and humans (Low and Donachie 1997). Listeriosis is considered a relatively rare disease, however, the infection has a high fatality rate of between 20-30% in humans (Newell, Koopmans et al. 2010). *L. monocytogenes* was first isolated and characterised in 1926 following an epidemic disease of rabbits and guinea-pigs in a laboratory breeding facility (Low and Donachie 1997). In 1981 the transmission of *L. monocytogenes* was linked with food following an outbreak implicating coleslaw (Warriner and Namvar 2009). Subsequent research indicated that *L.*

monocytogenes is naturally occurring in plant, soil, surface water, silage, sewage, slaughter house waste, milk and human and animal faeces (Farber and Peterkin 1991). The organism has also been isolated from animals including sheep, goats, cattle and poultry (Farber and Peterkin 1991). *L. monocytogenes* is gram positive, relatively resistant to acid and saline conditions, can be maintained in biofilms and can grow at a range of pH and temperatures, including under refrigeration temperatures (Roberts and Wiedmann 2003). These characteristics contribute to the adaptability and versatility of *L. monocytogenes* as a foodborne pathogen (Low and Donachie 1997). This adaptability means that the pathogen can survive in a range of environments, including food and food preparation surfaces. Although *L. monocytogenes* can grow on foods and processing surfaces, the pathogen favourably grows on floors, drains, processing equipment and in refrigerated rooms (Carpentier and Cerf 2011).

Since initial isolation and identification, *L. monocytogenes* has been shown to have a widespread distribution, with listeriosis outbreaks occurring across the world (Ramaswamy, Cresence et al. 2007). Such outbreaks occur following consumption of *L. monocytogenes*, through contaminated food or water (Ramaswamy, Cresence et al. 2007). Contamination can occur at various stages in the production line and the presence of *L. monocytogenes* in food processing environments and on equipment can contribute to the development of biofilms (Montañez-Izquierdo, Salas-Vázquez et al. 2012). The highly resistant nature of biofilms means that once established, organisms within the biofilm are likely to exist for extended periods of time, thus increasing the potential for contamination (Montañez-Izquierdo, Salas-Vázquez et al. 2012).

A wide range of foods have been implicated with *L. monocytogenes* poisoning including processed foods, meats, dairy products and foods consumed without much further heating or pasteurization such as ready to eat meals (Roberts and Wiedmann 2003). Although *L. monocytogenes* can be present in a variety of foods, it is most commonly found in raw meat, poultry and seafood (Farber and Peterkin 1991). The majority of *L. monocytogenes* contamination in meat is on the surface therefore, thorough cooking practises are essential however, there have been instances where *L. monocytogenes* has been present in animal muscle; in these cases cooking has not been sufficient to kill the pathogen (Farber and Peterkin 1991). As contamination of food products can occur at any stage during processing,

stringent measures must be present to prevent initial contamination and further spread of the organism (Ramaswamy, Cresence et al. 2007).

As *L.monocytogenes* is an opportunistic pathogen, pregnant women, neonates, immunocompromised individuals and those undergoing immuno therapy are at a higher risk than healthy individuals (Ramaswamy, Cresence et al. 2007). As the main defence of the body against *L. monocytogenes* is cell mediated immunity, individuals with limited T-cell functioning are prone to contracting listeriosis (Ramaswamy, Cresence et al. 2007). Consumption of foods contaminated with *L. monocytogenes* can cause febrile gastroenteritis or invasive systematic disease (Warriner and Namvar 2009). Symptoms associated with gastroenteritis include flu-like symptoms, abdominal pains and diarrhoea, these cases are generally not fatal (Warriner and Namvar 2009). However, contraction of the invasive disease is serious and generally affects vulnerable individuals (Warriner and Namvar 2009). In this instance the pathogen invades the gastro-intestinal epithelium or in the case of pregnant women the placental barrier, where the organism can become blood borne, spreading through the central nervous system and leading to fatal conditions including meningitis and encephalitis (Schlech and Acheson 2000). Early diagnosis and antibiotic treatment can reduce fatalities (Warriner and Namvar 2009).

The serious consequences associated with exposure to *L. monocytogenes* has resulted in significant efforts to prevent contamination during food processing (Schlech and Acheson 2000). Suggested methods of control include cleanliness in the food processing environment and use of disinfectants and chemical sanitizers (Schlech and Acheson 2000). Risk communication to susceptible members of the public is also considered an important control strategy (Warriner and Namvar 2009).

1.3.2. Shiga-toxin producing Escherichia coli

Escherichia coli was initially described in 1982 as part of the Enterobacteriaceae family (Mathusa, Chen et al. 2010). *E. coli* is a facultative anaerobic, rod-shaped, gram negative, non-spore forming bacterium, with some strains possessing peritrichous flagella (Mathusa, Chen et al. 2010). Certain strains of *E. coli*, including O157:H7 and O104:H21 have been identified as human pathogens (Mathusa, Chen et al. 2010). Studies completed in 1987 identified certain strains of enterohaemorrhagic *E. coli*, such as O157:H7 which have the ability to produce a cytotoxin capable of killing African green monkey Vero cells, the cytotoxin was subsequently named verotoxin (Fairbrother and Nadeau 2006). Verotoxin is

also referred to as Shiga toxin as further studies highlighted similarities between the verotoxin and the Shiga toxin produced by the organism *Shigella dysenteriae* (Bach, McAllister et al. 2002). Further work identified two different types of cytotoxin, which were recognized as SLT-I and SLT-II (Bach, McAllister et al. 2002). The over 100 serotypes of *E. coli* that produce one or both of these cytotoxins have been classified as Shiga-toxin producing *E. coli* (STEC), organisms that do not produce the Shiga-toxin are known as non-STEC (Bach, McAllister et al. 2002).

Since initial characterisation the main reservoirs of zoonotic *E. coli* have been identified as cattle and a number of other ruminants, which asymptomatically carry the pathogen and shed it through faeces (Bach, McAllister et al. 2002). From these reservoirs *E. coli* is transmitted to humans through consumption of food and water generally contaminated with animal faeces (Fairbrother and Nadeau 2006). Transmission may also occur through direct contact with infected animals or their immediate environment (Fairbrother and Nadeau 2006).

STEC outbreaks are generally associated with contaminated meat, especially lamb and ground beef, which is likely to be contaminated during the grinding process (Barlow, Gobius et al. 2006). Other foods implicated in outbreaks include dairy products such as milk, cheese, ice cream and produce such as lettuce, fruits and deli salads (Mathusa, Chen et al. 2010). *E. coli* is able to grow in a wide range of foods as the pathogen can survive in a range of conditions including acidic environments and temperatures ranging from 10˚C to 46˚C however, some strains have been shown to survive in temperatures as low as 6.5˚C (Farrokh, Jordan et al. 2013). The ability of the pathogen to grow in this range of conditions means that it is a significant risk factor in food processing environments. *E. coli* can enter the food processing chain at many points and can spread through inadequate food handling practises and contamination of foods and related surfaces (Rivas, Fegan et al. 2007). A further contributing factor in the persistence and spread of the organism is its ability to survive for extended periods of time in biofilms (Rivas, Fegan et al. 2007). The highly resistant nature of biofilms means that cells are dispersed during cleaning procedures or as cell replication occurs (Montañez-Izquierdo, Salas-Vázquez et al. 2012).

Although *E. coli* is commonly found in the intestinal tract of mammalian species, human consumption of the pathogen can result in severe illness (Fairbrother and Nadeau 2006). As

STEC are resistant to acidic conditions they are generally able to survive and attach to intestinal cells (Gyles 2007). Once attached, released Stx toxins bind to cells possessing the appropriate receptor, resulting in the death of those cells (Gyles 2007). The production of sufficient concentrations of the Stx toxins can result in damage to blood vessels in the colon and may cause watery or bloody diarrhoea (Gyles 2007). In severe cases the circulation of Stx toxins can lead to impaired kidney and neurological functioning, contributing to the development of potentially fatal conditions such as haemorrhagic colitis and haemolytic uremic syndrome, which can lead to acute or chronic renal failure (Hussein and Sakuma 2005). Other associated illnesses include strokes and nervous system abnormalities (Hussein and Sakuma 2005).

1.3.3 Pathogen prevention and surveillance

Reduction and prevention of foodborne illness is dependent on many factors including prevention of cross contamination by separating cooked and raw foods, careful food preparation and handling of raw products (Altekruse, Cohen et al. 1997). However, the increasingly widespread nature of foodborne disease makes the task of prevention large and multidisciplinary (Newell, Koopmans et al. 2010). Although good hygiene practises are essential in reducing cases of food related contamination and illness, especially in the domestic environment, there are cases when more stringent methods may be needed. Such methods include physical procedures such as cold plasma, ozone, gaseous sanitizers, ultra sound, ultra violet (UV) irradiation, high pressure processing and the chemical procedure of sanitation (Ramos, Miller et al. 2013).

The germicidal method of UV irradiation prevents microbial replication through initiating a reaction, resulting in the formation of thymine dimers. Although cells have repair mechanisms, exposure to high levels of UV irradiation can result in the disruption of cellular processes as there is an increased chance that dimers may be missed (Guerrero-Beltr and Barbosa-C 2004). Disinfection of surfaces, water and most food products occurs at a wavelength of 254nm (UV-C) as this wavelength is considered to have the optimal germicidal effect (Guerrero-Beltr and Barbosa-C 2004). The use of UV irradiation has been shown to be effective for foods including raw salmon fillets, ready to eat foods and fresh produce (Yaun, Sumner et al. 2004, Ozer and Demirci 2006, Chun, Kim et al. 2010). The advantages associated with the use of UV irradiation include that the method is simple, there are no remaining chemical residues and there is minimal change to sensory characteristics (Guerrero-Beltr and Barbosa-C 2004). However, the germicidal effect is only achieved when

light is applied in a direct line to the target, in some cases the light does not penetrate the target deeply enough (Guerrero-Beltr and Barbosa-C 2004). Other limitations include the high energy cost in New-Zealand, and the variation in effectiveness depending on the microbial species (Guerrero-Beltr and Barbosa-C 2004).

Another control procedure implemented to prevent pathogen growth is high pressure processing, also known as ultra- high pressure processing (Patterson 2005). The exposure of pressure levels between 30 and 500MPa can result in the denaturing of enzymes, disturbance of cell membranes and disruption to the processes of DNA replication and protein synthesis (Smelt 1998). Although exposure to high pressure damages or kills cells due to the disruption of vital cell processes, bacterial spores cannot be inactivated by high pressure alone (Smelt 1998). Further to this, the structural and sensory quality of the food may be altered and the success of pressure based treatments is dependent on factors including properties of the food source, temperature, water content and characteristics of the target microbe (Patterson 2005).

A more commonly used control procedure is the use of chemical sanitizers such as chlorine, which disrupts bacterial cell walls and membranes through the oxidation of the thiol group, thus resulting in cell damage or death (WHO 1998). Although chlorine has been widely used, growing environmental and health concerns have prompted the use of alternative sanitizers such as hydrogen peroxide, peroxyacetic acid and acidified sodium chlorite (Olaimat and Holley 2012). These products have been shown to be effective on fresh produce, as they reduce and maintain pathogen populations after treatment, even at abusive temperatures (Abadias, Alegre et al. 2011). However, surface imperfections, biofilm formation and bacterial harbourage in leaf pores are all drawbacks associated with sanitizer use (Olaimat and Holley 2012).

Other procedures used to control microbial populations on foods and related surfaces include electric pulse fields and pulsed light (Dunn, 1996). The method of electric pulse fields kills vegetative microbes by storing electrical energy in a high density electrical storage capacitor (Dunn, 1996). The release of this energy in short, high intensity pulses generates high power levels, killing bacterial cells (Dunn, 1996). The specific mechanism responsible for microbial inactivation has not been established as yet (Wouters, Alvarez et al. 2001). However, previous studies have suggested that the application of light pulses onto microbial

cells causes electroporation, or the formation of pores in the membrane resulting in significant damage, such that the cell cannot recover (Wouters, Alvarez et al. 2001). The use of this method has been effective in products including milk, liquid eggs, juices, emulsions and food ingredients such as flavours and protein concentrates (Dunn, 1996). The advantage of using electric pulse fields to eliminate microbial populations include that the procedure can be applied at a range of temperatures where no thermal damage can occur, thus retaining the original taste, colour, texture and functionality of products. However, the method is not considered suitable for inactivating bacterial spores, inactivation is dependent on the strain of the microbe and the method is more suited towards products with a low pH (Wouters, Alvarez et al. 2001).

The method of pulsed light has been shown to kill vegetative bacteria, protozoan oocytes, fungal spores and viruses by emitting intensive white light onto surfaces and foods (Dunn, 1996). The pulses of light emitted range from far UV to viable to infrared. Studies have indicated that the method can be successfully applied onto foods including vegetables, fresh fruits, poultry, fish, eggs and certain liquids (Dunn, 1996). The advantages associated with the use of the method include that the process is rapid and only a few flashes of light are required to kill existing microbes. However, limitations include sample heating, either from light adsorption or the lamp which may restrict the effectiveness of the treatment (Gomez-Lopez, Ragaert et al. 2007). As well as this, for inactivation to occur photons from the light must directly hit the cell any blockages will impair treatment and opaque foods or surfaces will only be superficially treated (Gomez-Lopez, Ragaert et al. 2007).

Despite the implementation of these robust methods reported cases of foodborne illness across the world continue to increase each year, prompting research into alternative methods of pathogen control, one such alternative is the use of phages as a biocontrol agent (Hagens and Loessner 2007).

1.4 Characteristics of Phages

1.4.1 Bacteriophages

Bacteriophage (phages) are a group of viruses that specifically infect bacterial cells, resulting in cell lysis or lysogeny (Sulakvelidze, Alavidze et al. 2001). Phages are obligate parasites, with genomes consisting of either DNA or RNA, this genomic material is surrounded by a capsid protein which can be enveloped, especially in the case of viruses that bud off cells (Marsh and Helenius 2006). Some phages also have a contractile tail which is associated with the transfer of viral genetic material or in some cases enzymes into the host cell to induce an infection (Hanlon 2007). Once the viral genome is within the host cell, phage replication will occur and the cell may go through either the lytic or the lysogenic replication cycle depending on the type of phage.

Of all microbial groups, phages are considered the most abundant, with an estimated 10^{32} phages on the planet (Hanlon 2007). Studies have suggested that phage infections are responsible for 20-50% of bacterial mortality, although infections do not decrease bacterial numbers (Maura and Debarbieux 2011). However, local variation in numbers has been observed, suggesting co-evolution between bacteria and phages (Maura and Debarbieux 2011). The success of phages is attributed to many factors especially the generation and maintenance of diversity which is dependent on factors such as evolution, adaptation, environmental heterogeneity and trophic interactions (Jessup and Forde 2008). Phages are classified into families based on morphology and genome characteristics (Table 1.2), however, a number remain unassigned (King, Adams et al. 2011)

Due to the ability of phages to infect and kill bacterial cells, phage therapy is considered an option to combat bacterial infections prior to the emergence and widespread administration of antibiotics (Sulakvelidze, Alavidze et al. 2001). Phage therapy declined in popularity before the emergence of antibiotics due to false advertising of phage related products, single phage preparation for multiple bacteria types and importantly, an incomplete understanding of the heterogeneity and ecology of bacteria and phage (Atterbury 2009). However, with the current global rise of antibiotic and chemical resistance, phage therapy and pathogen biocontrol are regaining popularity and priority.

Table 1.2: Classification of phages by morphology and genome characteristics (Sharp 2001, Ackermann 2003, Abedon 2009, Abedon 2011, King, Adams et al. 2011).

Order	Family	Morphology	Nucleic Acid	Genome Size (kb)	Nucleic acid strands	Genome structure	Characteristics
Caudovirales	*Myoviridae*	Tailed	DNA	34-240	Double	Linear	Contractile tails
	Siphoviridae		DNA	22-121	Double	Linear	Long tail, non-contractile
	Podoviridae		DNA	16-70	Double	Linear	Short tail
Unassigned	*Microviridae*	Polyhedral	DNA	4.5 – 5.5	Single	Circular	
	Tectiviridae		DNA	15	Double	Linear	Double capsid
	Corticoviridae		DNA	9-10	Double	Circular	Superhelical, complex capsid. Contains lipids.
	Leviviridae		RNA	3.5 – 4.2	Single	Linear	
Unassigned	*Cystoviridae*	Filamentous	RNA	13	Double	Linear, segmented	Enveloped, lipids
Ligamenvirales	*Lipothrixviridae*		DNA	16	Double		Infects Archea
Unassigned	*Inoviridae*		DNA	4.5-8.5	Single	Circular	Filamentous or rods
	Reoviridae		DNA	1-4	Double	Linear	Infects Archea
Unassigned	*Fuselloviridae*	Pleomorphic	DNA	14.5-15.5	Double	Circular	Infects Archea. Lemon shaped.
	Plasmaviridae		DNA	12	Double	Circular	Spindle shaped, no capsid.

1.4.2 History of phages

Phages were first independently discovered by Fredrick Twort and Felix d'Herelle, in 1915 and 1917 respectively (Monk, Rees et al. 2010). However, their presence was first documented in 1896 by Ernest Hankin, a British bacteriologist who reported observing a filterable antibacterial activity against the organism *Vibrio cholerae* in the rivers Ganges and Jumna in India (Sulakvelidze, Alavidze et al. 2001). These observations suggested the

presence of an unidentified entity which passed through filters, was heat liable and limited the spread of cholera, thus cholera epidemics (Sulakvelidze, Alavidze et al. 2001). Two years later the Russian bacteriologist Gamalea reported similar observations against *Bacillus subtilis,* however these observations were not further investigated until the work of Fredrick Twort, a medically trained English bacteriologist (Duckworth 1976). Twort suggested that the observed phenomenon was due to the presence of a virus, however, this hypothesis was not further pursued (Duckworth 1976). Two years later phages were officially discovered by Felix d'Herelle, a French Canadian microbiologist (Keen 2012). d'Herelles discovery came from the observation of small, clear plaques on an agar medium containing stool samples from dysentery patients (Summers 2001). As these plaques appeared to kill bacteria in the surrounding area, d'Herelles coined the name bacteriophage, meaning "bacteria" and "*phagein*" to "eat" or "devour"(d'Herelle 1917).

1.4.3 Phage Lifecycle

Phages have two lifecycles, the lytic lifecycle and the lysogenic lifecycle (Figure 1.1). Any phage lifecycle begins with the complex process of attachment and it is through advances in technology that insights into this process have been gained (Bertin, de Frutos et al. 2011). Initial infection of the host begins when the phage binds to a specific receptor located on the host surface (Marsh and Helenius 2006). The binding interaction between a phage receptor binding protein (RBP) and a surface component of the host cell, results in the production of a signalling cascade which triggers the processes required for viral entry (Marsh and Helenius 2006). Studies have indicated that in phage with a viral tail, for example T2, initial attachment is followed by contraction of the cell wall and viral tail, thus bringing the phage closer to the host cell before injecting the genome (Cota-Robles and Coffman 1963). The genome then travels into the host cell, leaving an empty phage particle attached to the host surface (Bertin, de Frutos et al. 2011). This attachment process appears to be general across phage families however; the mechanisms by which genomes are internalized within hosts differ between phages (Rakhuba, Kolomiets et al. 2010).

1.4.3.1 Lytic pathway

Activation of the lytic pathway results in a number of processes which lead to the development and accumulation of newly synthesised viral particles (Echols 1972). Following viral synthesis, rapid cell lysis occurs resulting in the release of progeny phage (Echols 1972). The lytic cycle begins when a phage comes into contact with a bacterial cell through random motion (Hanlon 2007). This contact is followed by initial attachment or adsorption to

bacterial surface receptor sites, which include proteins, teichoic acid and peptidoglycan (Marsh and Helenius 2006). The adsorption rate between the phage and host cell is dependent on the concentration of bacteria and phage present (Puck, Garen et al. 1951). However, the addition of divalent ions has been shown to promote attachment between phage and bacteria through increasing the strength of the electrostatic forces between cells (Puck, Garen et al. 1951).

This initial stage of attachment is reversible however, attachment soon moves to an irreversible state and is followed by the transfer of the viral genome into the bacterial host (Maura and Debarbieux 2011). The viral genome is transferred to the host through many routes, the most common being the contraction of the tail through a pore formed on the host cell surface (Hanlon 2007). Once inside the host cell the viral genome is replicated through the action of host cell machinery (Echols 1972). This process results in the redirection of host cell machinery and metabolic processes to synthesise the components required for viral assembly (Hanlon 2007). Synthesis involves the development of proteins required for the head and tail structures and coating of the genome (Echols 1972). Once viral components have been synthesised and assembled they are released through cell lysis which occurs when enzymes such as holin and endolysin are released, disrupting the cell membrane and resulting in the degradation of bacterial peptinoglycan (Matsuzaki, Rashel et al. 2005).

1.4.3.2 Lysogenic pathway

Phages replicating through the lysogenic pathway are known as temperate (Hanlon 2007). In this form of replication the lytic functions of the virus are turned “off” and the host cell survives (Echols 1972). Like the lytic cycle, initial contact, attachment and transfer of phage genetic material occurs however, during the lysogenic cycle the phage genome is either integrated into host cell DNA or exists episomally (Matsuzaki, Rashel et al. 2005). The process of integration occurs through site-specific recombination at specific regions of both the host and phage genomes (Matsuzaki, Rashel et al. 2005). Bacterial cells that have integrated a phage genome are known as lysogenic (Hanlon 2007). As the host cell genome is replicated the phage genome is also replicated and daughter cells each receive a copy, these sequences are termed prophages (Hanlon 2007). Temperate phages remain successful by preventing other phages from infecting the host cell, by repressing the genes responsible for lytic replication through direct repression or alteration of host cell wall receptor sites (Labrie and Moineau 2007, Labrie, Samson et al. 2010).

Although prophages can replicate through the lysogenic cycle for many generations, exposure of the host cell to stressful conditions such as UV irradiation, mutagenic agents (DNA damage), pressure and osmotic and nutrient stress can result in the activation of the lytic cycle, cell lysis and liberation of progeny phage (Hanlon 2007, Rice and Bayles 2008). Temperate phage replication does not result in the release of many progeny phage, and the majority of phage produced are from a small percentage of the stressed host cells (Lwoff 1953). For this reason temperate phage are not considered suitable for biocontrol purposes (Matsuzaki, Rashel et al. 2005).

In addition to the lytic and lysogenic lifecycles, an alternate lifecycle, pseudolysogeny has been described (Ripp and Miller 1998). Pseudolysogeny is defined as an unstable state whereby the phage genome co- exists with the bacterial host for extended periods of time (Ripp and Miller 1998). In the pseudolysogenic state the phage genome does not actively replicate through the lytic or lysogenic pathways, instead it enters a dormant state and is maintained within the host cell (Ripp and Miller 1998). Phages have been observed to enter the pseudolysogenic state when nutrients are limiting for the host and when environmental conditions improve, the phage begins to actively replicate through either the lytic or lysogenic cycle (Ripp and Miller 1998).

Some phages may also kill bacterial cells through a phenomenon known as lysis from without (Abedon 2011). Lysis from without occurs when a high number of phages simultaneously infect a single bacterial cell, causing destruction of the bacterial cell through destabilization of the cell membrane integrity (Monk, Rees et al. 2010). This process results in the rapid death of bacterial cells and does not require active phage replication (Abedon 2011).

1.4.4 Natural existence of phages

The abundance of phages was initially highlighted through aquatic studies, which suggested that phages must continually infect their hosts to renew their population (Maura and Debarbieux 2011). As phages are mediators in ecological and industrial processes and have been co-evolving with their hosts for billions of years, they are isolated from environments inhabited by their host (Greer 2005). Such environments include soil, sewage, water, farm and processing plants, effluent, faeces and retail foods (Greer 2005). Certain phages have been co-evolving with their hosts for billions of years therefore, they are generally isolated

from environments inhabited by their host (Hanlon 2007). Despite their essential role in ecosystem functions, little is known about phage genetic diversity (Roux, Hallam et al. 2015).

Research into phage characteristics and genomics has indicated that phages are more diverse than previously thought (Hatfull 2008). Although phage genomes are relatively small, sequenced isolates show limited similarity to previously sequenced genomes, with many sequences identified as uncharacterised (Roux, Hallam et al. 2015). The sequencing of phage genomes has highlighted three common themes (Hatfull 2008). Firstly, a high degree of genetic diversity within genomes suggests an early evolutionary origin (Hatfull 2008). Secondly, genomes appear to have a mosaic pattern, indicating significant levels of horizontal gene transfer throughout evolutionary time (Hatfull 2008). Studies have also suggested that phages may have a role in lateral gene transfer between environments (Sano, Carlson et al. 2004). Thirdly, a number of sequences within genomes have unknown functions, highlighting the need for further research (Roux, Hallam et al. 2015).

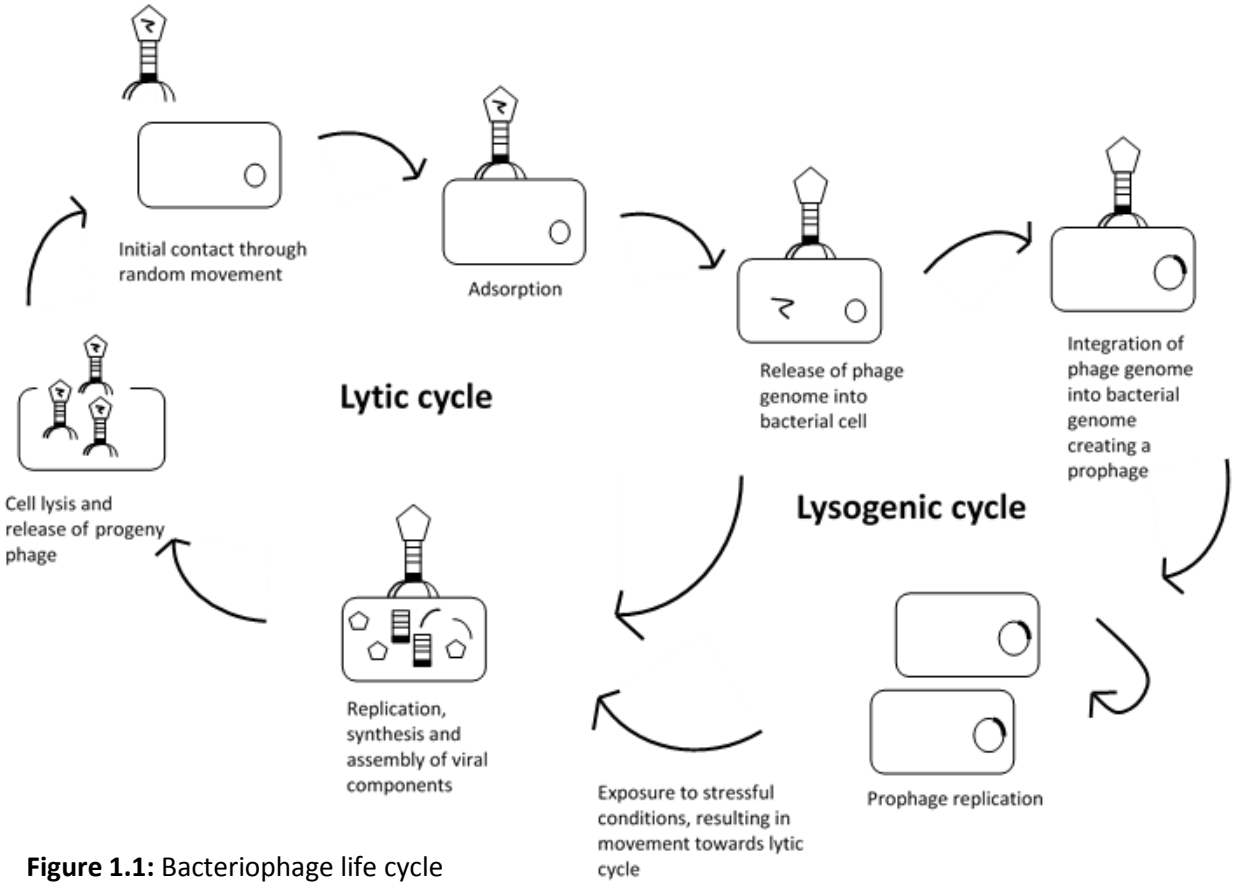

Figure 1.1: Bacteriophage life cycle

1.5 Phage as Biocontrol Agents

Despite modern production systems and intensive food monitoring approaches, incidences of foodborne disease continue to increase worldwide (Hagens and Loessner 2007). This trend is thought to result from mass production and globalization, which results in foodborne contamination and illness on a larger scale (Hagens and Loessner 2007). The use of existing interventions has not been effective in controlling pathogen contamination and spread of foodborne pathogens therefore, alternative strategies are required (Garcia, Martinez et al. 2008). One such alternative is the use of phages as a biocontrol agent (Hagens and Loessner 2007).

1.5.1 Phage characteristics

For phages to be considered as candidates for biocontrol in the food industry they should possess a number of characteristics which allow for a high potential to attach and kill bacteria and a low potential to cause harm to the environment (Loc-Carrillo and Abedon 2011).

Firstly, the selected candidate phages should ideally replicate only through the lytic cycle (Henry and Debarbieux 2012). Lytic phage replication ensures that progeny phage are released at a rapid rate therefore, surrounding bacterial populations can thus be infected resulting in a rapid decline of the pathogen load (Henry and Debarbieux 2012). The use of obligately lytic (virulent) phages also limits the possibility that the phage is carrying genes associated with toxicity, thus reducing the potential for environmental harm (Loc-Carrillo and Abedon 2011). Further to this, the ability of a phage to be used in a specific food system, the potential for transduction and antibiotic resistance should be carefully evaluated, through characterisation experiments prior to application (Hagens and Loessner 2014).

An understanding of phage host range is important as some phages are highly specific to a bacterial strain whereas, others have the ability to infect bacteria within the same genus (Strauch, Hammerl et al. 2007). For example the *Listeria* phage A511 has a broad host range and infects and kills *L. monocytogenes* of different serotypes as well as strains of other *Listeria* species, making it an ideal phage for biocontrol (Kim, Siletzky et al. 2008). Host range information provides an understanding of the types of bacteria that can be infected and ensures that there are limited negative effects on natural microflora (Mahony, McAuliffe et al. 2011). The efficacy of phages at the temperature at which they will be used should be

considered due to the temperature-dependent nature of the phage-host interaction (Mahony, McAuliffe et al. 2011).

1.5.2 Advantages of phage use

As phages have co-existed with bacteria for billions of years they have a significant role in the control of naturally occurring bacterial populations (Hagens and Loessner 2014). A crucial factor in phages success is host specificity, phages infect and lyse bacterial cells only, therefore they are safe and harmless to use against mammalian cells (Garcia, Martinez et al. 2008). Phages recognize their host through specific binding receptors found on the surface of the bacterial cell (Heller 1992). This specificity makes it unlikely that phages will infect cells other than their host (Hagens and Loessner 2007). As phages are abundant in the environment it is suspected that they are regularly consumed through food and water, this has not resulted in undesirable effects suggesting that limited phage consumption is harmless (Monk, Rees et al. 2010). Further to this, studies in animal and human models have not resulted in adverse effects following phage consumption (Bruttin and Brüssow 2005, Hagens and Loessner 2007).

As well as this, phages are self-limiting, low or single phage doses will result in continued multiplication as long as there is a host threshold present, increasing the possibility that pathogen concentrations will be significantly minimised (Sillankorva, Oliveira et al. 2012). Phages also have a low inherent toxicity as they are composed mainly of nucleic acid and proteins, making them safer than other alternatives (Loc-Carrillo and Abedon 2011). Other advantages include that phages are cheap and easy to isolate and propagate, can generally withstand food environmental pressures and have proved to have an extended shelf life (Sillankorva, Oliveira et al. 2012).

Phages can also be applied from the beginning to the end of a food production process (Garcia, Martinez et al. 2008). For example, purified high-titre phage lysates have been used for species-specific control of bacteria during pre and post-harvest phases of food production and storage (Greer 2005). They can also be used to prevent initial colonization, control disease in livestock, as a decontamination tool for carcasses and raw products including fresh fruit and vegetables and as a disinfectant for surfaces (Garcia, Martinez et al. 2008). The relatively simple handling of phages and the widespread applications in combination with other methods has the potential to decrease bacterial contamination and therefore, spread (Garcia, Martinez et al. 2008).

1.5.3 Limitations of phage use

Despite the advantages associated with using phage as a biocontrol tool, there are limitations to be considered. Firstly, direct phage exposure has the potential to result in the development of bacterial resistance or cross resistance mechanisms (Hudson, McIntyre et al. 2010, Anany, Chen et al. 2011). As bacteria multiply rapidly they are likely to develop resistance mechanisms against the phages they are exposed to. However, the host specific nature of phages suggests that they will adapt and develop mechanisms to overcome this resistance (Hagens and Loessner 2007). Other potential solutions to this problem include the use of phage cocktails, the regular disinfecting of equipment and surfaces and the use of immobilized phage which prevent phage wastage by ensuring that applied phages are retained near the treated surface (Anany, Chen et al. 2011).

A second limitation to phage use is lysogenic conversion which can result in the mutation of phages from a lytic cycle to a lysogenic cycle (Greer 2005). In some cases the phage genes integrated into the bacterial genome may include moron genes, which can be transcribed and have the potential to phenotypically alter bacteria, which may increase host virulence (Hagens and Loessner 2010). However, there is no evidence to suggest that this transfer and conversion phenomenon has occurred in food systems (Greer 2005). Other limiting factors include diffusion rates that may decrease the chance of host-phage collisions, microbial load which may act as a barrier by providing unspecific phage binding sites, the requirement for a threshold number of bacterial targets and other external conditions including temperature, water activity, pH and inhibitory compounds (Garcia, Martinez et al. 2008).

1.5.4 Future of phage biocontrol

Advances in technology have provided a means by which more information can be gathered and applied to phage biocontrol. For example, advances in the field of next generation sequencing have allowed for full genome sequences to be easily obtained and applied to various systems, this is useful for evaluating the suitability of phages for food application systems (Mahony, McAuliffe et al. 2011). Further advances in phage application include the use of single gene products such as isolated proteins and enzymes, rather than the entire phage particle (Hagens and Loessner 2014). The use of such products would remove difficulties associated with infective particles and would calm fears of genetic recombination and instability, while still maintaining phage specificity (Henry and Debarbieux 2012). There are currently two commercially available *Listeria* phage products, Listex 100 and ListShield, both of which have been granted approval by the FDA and USDA (Hagens and Loessner

2014). Studies investigating both products have highlighted their effectiveness on various food products and working surfaces (Hagens and Loessner 2014).

1.6 Phage Activity on Food and Food Preparation Surfaces

To investigate the efficiency of phages in controlling bacterial populations on foods and related surfaces a number of studies have been completed. One such study used the *Listeria* phages A511 and P100 to evaluate efficiency in solid and liquid ready to eat foods (Guenther, Huwyler et al. 2009). All samples were spiked with *L. monocytogenes* before phage addition and incubation for six days at 6°C. Results of the study indicated that bacterial counts on solid and liquid foods decreased to below the level of direct detection following phage addition. These results highlighted the importance of intrinsic factors such as ionic strength, pH and chemicals which may interfere with phage and bacterial attachment. Such factors are largely dependent on the initial nature of the food and changes that occur throughout storage. The results also emphasised the importance of initial phage concentration at the time of application. Application of a higher concentration of phages during the initial stage generally results in greater inactivation of the bacterial population however, the concentration of phages added should be optimized depending on the type of food system to which it is applied. As well as this, the probability of phage infecting a cell in a given unit area, co-location of the host and phage and diffusion of the phage on a surface should be considered prior to phage application (Hudson, McIntyre et al. 2010). The study concluded that the phages A511 and P100 were virulent, have a broad host range and are effective as biocontrol agents against *L. monocytogenes.*

In a similar study a cocktail of three previously isolated and characterised *E. coli* phages were applied to ground beef, spinach and cheese contaminated with O157:H7 *E. coli* (Hong, Pan et al. 2014). The study aimed to investigate whether phage application would reduce bacterial concentrations. The results of the study indicated that the addition of the phage cocktail to ground beef decreased bacterial numbers when undercooked, refrigerated and stored at room temperature. Similar results were obtained following phage application to spinach with numbers decreasing when stored at room temperature for 24, 48 and 72 hours. However, the application of the cocktail to contaminated cheese did not reduce bacterial numbers. Researchers also isolated and characterised three phage resistant *E. coli* O157:H7 strains. Together, these results suggest that phages can be used as a method of biocontrol, however more studies considering phage resistance and resistance strategies are required to enhance the use of phage based products.

Although foods and related products can be contaminated in a number of ways a significant threat in the food industry is the formation of biofilms (Kumar and Anand 1998). Biofilms are defined as groups of single or multi-species surface associated microbial cells which are enclosed in an extracellular polymeric substance (Donlan 2002). The first stage in biofilm formation is attachment which occurs when bacterial cells come into contact with a surface. The process of attachment is mediated by a number of factors including motility and structure of the bacterial cell, nature of substratum, surface roughness and the presence of liquids (Frank 2001). These factors influence initial adhesion thus, further attachment of a bacterial cell onto a surface (Fletcher and Pringle 1985). Although these properties are crucial in the process of surface attachment, environmental and growth conditions generally determine the strength of adhesion and subsequent attachment (Fletcher and Pringle 1985). Following initial contact bacterial cells release exopolysaccharides which are crucial in establishing and maintaining surface attachment (Kumar and Anand 1998). The presence of established biofilms in food processing environments is a significant contamination threat as once established, biofilms may disperse daughter cells into the surrounding environment further to this, biofilms are highly resistant to cleaning products and chemicals therefore, they are increasingly difficult to remove once irreversible attachment has occurred (Kumar and Anand 1998).

A study conducted by Jassim *et al* (2012) used a phage cocktail consisting of 140 lytic *E. coli* phages on naturally and artificially contaminated food samples and biofilms. The cocktail had been previously designed, bred and optimised through non-genetic methods to develop a cocktail specific to a wide range of *E. coli* strains. Application of the cocktail resulted in a significant reduction of *E. coli* cells found on both naturally and artificially contaminated samples and application onto biofilms resulted in a three log reduction and disintegration of the biofilm matrix. Although total removal of the biofilm did not occur, this result suggests that there is potential for phage biocontrol to be used against contaminated foods and biofilms (Jassim, Abdulamir et al. 2012).

1.7 Methods of *Listeria* and *E. coli* Phage Isolation

The general method used for phage isolation is the plaque assay or overlay method, however due to the diversity of samples from a wide range of environments, specific isolation protocols may vary (Kropinski, Mazzocco et al. 2009). In most cases divalent ions, such as Ca^{2+} and Mg^{2+} are added to overlay assays as their presence strengthens

electrostatic forces, thus increasing the potential for phage adsorption and infection (Shafia and Thompson 1964, Van Twest and Kropinski 2009)

1.7.1 Listeria phage isolation

Listeria phages have been isolated from environments where their host is present such as soil, wastewater, sewage and foods, as well as seafood processing plants and silage (Beuchat 1996, Vongkamjan, Switt et al. 2012, Arachchi, Mutukumira et al. 2013).

Although a significant amount of research has been conducted to investigate the presence of *Listeria* phages in the environment, studies considering the presence and characteristics of phages in processing environments are rare. Therefore, a study was conducted by Kim, Siletzky, & Kathariou (2008) to investigate the host range of *Listeria* phages isolated from turkey processing plants in the United States. Following initial processing of swab samples, phages were isolated and subsequently tested for host range using the overlay method with the addition of calcium chloride. The results of their study suggested that the majority of phages isolated had a broad host range and were specific to the *Listeria* species present in the processing plant (Kim, Siletzky et al. 2008).

In another study, silage samples were collected from dairy farms and processed in order to gain a better understanding of the ecology and diversity of *Listeria* phages present in the environment. Following purification and further experimentation researchers were able to conclude that *Listeria* phages were abundant in the farm environment, had a broad host range and high levels of genomic diversity (Vongkamjan, Switt et al. 2012).

1.7.2 E. coli Phage isolation

E. coli phages have been isolated from a variety of foods including fruits, vegetables, dairy and meat products that have been infected with high levels of the host organism (Barlow, Gobius et al. 2006).

One study involved the isolation of phages from bovine and ovine faecal matter. After initial phage isolation, characterisation experiments were conducted to further investigate the properties of the isolated phages. The results of the study indicated that isolated phages had the ability to lyse O157 cultures, but not non-O157 and non-*E. coli* cultures, furthermore bacterial cells that expressed a truncated lipopolysaccharide or did not express the O157 antigen were not susceptible to phage infection. Researchers concluded that the isolated

phages had the potential to be used as biocontrol tools for animals and foods without compromising food quality or naturally occurring microflora (Kudva, Jelacic et al. 1999).

In an additional study the *E. coli* phage φ241 was isolated and characterised. φ241 was isolated from industrial fermented cucumber, the environment was highly acidic with a pH less than 3.7, and a salinity level less than 5% (Lu and Breidt 2015). The results of the characterisation studies suggested that φ241 was specific to the O157 antigen, as *E. coli* strains lacking the antigen were resistant to infection. Researchers concluded that φ241 is suitable for the purpose of biocontrol as the phage showed lytic ability, was able to function in high acid and salt conditions and was specific to the target host.

The results of such studies provide an essential insight into the presence and characteristics of *Listeria* and *E. coli* specific phages isolated from industrial and farming environments. Furthermore, these results contribute to existing information regarding the application of phages as a method of biocontrol.

1.8 Conclusion

The serious and widespread nature of foodborne diseases has prompted the implementation of intensive controls aimed at reducing the spread of foodborne pathogens and associated outbreaks. However, as incidences of foodborne illness continue to increase worldwide alternative methods of biocontrol, including phages are being considered. Phages have a number of favourable characteristics including host specificity, easy handling, good safety profile and widespread applications. Phages as a biocontrol tool have the potential to reduce contamination, thus incidences of foodborne illness worldwide. However, for phages to be successfully implemented in commercial environments research into their characteristics and application must continue.

1.9 Research Objectives

The aim of this research was firstly, to isolate *Listeria* and *E. coli* phages from faecal matter, wastewater samples and dairy swabs. Previously and newly isolated phages were then characterised based on their morphology and kinetics through adsorption assays, transmission electron microscopy and host range analysis. Phages showing biocontrol potential were further investigated through applied experiments. *Listeria* phages were tested on biofilms containing *Listeria*, and *E. coli* phages were applied onto cooked roast beef samples to investigate potential for application in industrial settings.

1.10 Hypotheses

The hypotheses of this study were as follows:

- That phages have the potential to be used as a biocontrol tool in foods, as they are naturally occurring infectious agents of bacteria. Their application on *E. coli* attached to cooked roast beef, and *Listeria* in biofilms is expected to result in a decrease in the proportion of live bacterial cells on a surface.
- The abundant nature of phages suggests that there is a likely correlation between the presence of phages and their bacterial hosts in the environment. Therefore, it is likely that phages will be present in faecal matter, wastewater and dairy samples, and can be isolated from such samples using standard phage isolation and purification methods.
- That characterisation and application experiments conducted in the lab are indicative of the biocontrol potential of phages when applied in commercial food production systems.

1.11 References:

Abadias, M., et al. (2011). "Evaluation of alternative sanitizers to chlorine disinfection for reducing foodborne pathogens in fresh-cut apple." Postharvest Biology and Technology **59**(3): 289-297.

Abedon, S. T. (2009). "Phage evolution and ecology." Advances in applied microbiology **67**: 1-45.

Abedon, S. T. (2011). "Lysis from without." Bacteriophage **1**(1): 46-49.

Acheson, D. and A. E. Fiore (2004). "Hepatitis A transmitted by food." Clinical Infectious Diseases **38**(5): 705-715.

Ackermann, H.-W. (2003). "Bacteriophage observations and evolution." Research in Microbiology **154**(4): 245-251.

Adams, M. H. (1959). "Bacteriophages." Bacteriophages.

Altekruse, S., et al. (1997). "Emerging foodborne diseases." Emerging infectious diseases **3**(3): 285.

Anany, H., et al. (2011). "Biocontrol of Listeria monocytogenes and Escherichia coli O157: H7 in meat by using phages immobilized on modified cellulose membranes." Applied and environmental microbiology **77**(18): 6379-6387.

Arachchi, G., et al. (2013). "Characteristics of three listeriaphages isolated from New Zealand seafood environments." Journal of applied microbiology **115**(6): 1427-1438.

Atterbury, R. (2009). "Bacteriophage biocontrol in animals and meat products." Microbial biotechnology **2**(6): 601-612.

Bach, S., et al. (2002). "Transmission and control of Escherichia coli O157: H7-A review." Canadian Journal of Animal Science **82**(4): 475-490.

Barlow, R. S., et al. (2006). "Shiga toxin-producing Escherichia coli in ground beef and lamb cuts: results of a one-year study." International journal of food microbiology **111**(1): 1-5.

Bertin, A., et al. (2011). "Bacteriophage–host interactions leading to genome internalization." Current opinion in microbiology **14**(4): 492-496.

Beuchat, L. R. (1996). "Listeria monocytogenes: incidence on vegetables." Food Control **7**(4): 223-228.

Bruttin, A. and H. Brüssow (2005). "Human volunteers receiving Escherichia coli phage T4 orally: a safety test of phage therapy." Antimicrobial agents and chemotherapy **49**(7): 2874-2878.

Carpentier, B. and O. Cerf (2011). "Review—Persistence of Listeria monocytogenes in food industry equipment and premises." International journal of food microbiology **145**(1): 1-8.

Chun, H.-H., et al. (2010). "Inactivation of foodborne pathogens in ready-to-eat salad using UV-C irradiation." Food Science and Biotechnology **19**(2): 547-551.

Cota-Robles, E. and M. Coffman (1963). "Fine structure of the bacteriophage attachment process." Journal of bacteriology **86**(2): 266-273.

Cressey, P. and R. Lake (2011). "Estimated incidence of foodborne illness in New Zealand: Application of overseas models and multipliers." Report—New Zealand Government, Christ Church, New Zealand.

d'Herelle, F. (1917). "An invisible microbe that is antagonistic to the dysentery bacillus." Comptes rendus Acad. Sci. Paris **165**: 373-375.

Delbrück, M. (1940). "The growth of bacteriophage and lysis of the host." The journal of general physiology **23**(5): 643.

Donlan, R. M. (2002). "Biofilms: microbial life on surfaces." Emerg Infect Dis **8**(9).

Duckworth, D. H. (1976). "" Who discovered bacteriophage?"." Bacteriological reviews **40**(4): 793.

Dunn, J. (1996). “Pulsed light and pulsed electric field for foods and eggs.” Poultry Science **75**(9): 1133-1136.

Echols, H. (1972). "Developmental pathways for the temperate phage: lysis vs lysogeny." Annual review of genetics **6**(1): 157-190.

Fairbrother, J. and E. Nadeau (2006). "Escherichia coli: on-farm contamination of animals." Rev Sci Tech **25**(2): 555-569.

Farber, J. and P. Peterkin (1991). "Listeria monocytogenes, a food-borne pathogen." Microbiological reviews **55**(3): 476.

Farrokh, C., et al. (2013). "Review of Shiga-toxin-producing Escherichia coli (STEC) and their significance in dairy production." International journal of food microbiology **162**(2): 190-212.

Fletcher, M. and J. H. Pringle (1985). "The effect of surface free energy and medium surface tension on bacterial attachment to solid surfaces." Journal of Colloid and Interface Science **104**(1): 5-14.

Frank, J. F. (2001). "Microbial attachment to food and food contact surfaces." Advances in food and nutrition research **43**: 320-357.

Gallet, R., et al. (2011). "Effects of bacteriophage traits on plaque formation." BMC microbiology **11**(1): 181.

Gallet, R., et al. (2009). "High adsorption rate is detrimental to bacteriophage fitness in a biofilm-like environment." BMC evolutionary biology **9**(1): 241.

Garcia, P., et al. (2008). "Bacteriophages and their application in food safety." Letters in applied microbiology **47**(6): 479-485.

Gomez-Lopez, V. M., et al. (2007). "Pulsed light for food decontamination: a review." Trends in Food Science & Technology **18**(9): 464-473.

Greer, G. G. (2005). "Bacteriophage control of foodborne bacteria." Journal of Food Protection® **68**(5): 1102-1111.

Guenther, S., et al. (2009). "Virulent bacteriophage for efficient biocontrol of Listeria monocytogenes in ready-to-eat foods." Applied and environmental microbiology **75**(1): 93-100.

Guerrero-Beltr, J. and G. Barbosa-C (2004). "Advantages and limitations on processing foods by UV light." Food science and technology international **10**(3): 137-147.

Gyles, C. (2007). "Shiga toxin-producing An overview." Journal of animal science **85**(13_suppl): E45-E62.

Hagens, S. and M. J. Loessner (2007). "Application of bacteriophages for detection and control of foodborne pathogens." Applied Microbiology and Biotechnology **76**(3): 513-519.

Hagens, S. and M. J. Loessner (2010). "Bacteriophage for biocontrol of foodborne pathogens: calculations and considerations." Current pharmaceutical biotechnology **11**(1): 58-68.

Hagens, S. and M. J. Loessner (2014). "Phages of Listeria offer novel tools for diagnostics and biocontrol." Frontiers in microbiology **5**.

Hall, G., et al. (2008). "Foodborne Illnesses: Overview."

Hanlon, G. W. (2007). "Bacteriophages: an appraisal of their role in the treatment of bacterial infections." International journal of antimicrobial agents **30**(2): 118-128.

Hatfull, G. F. (2008). "Bacteriophage genomics." Current opinion in microbiology **11**(5): 447-453.

Heller, K. J. (1992). "Molecular interaction between bacteriophage and the gram-negative cell envelope." Archives of microbiology **158**(4): 235-248.

Henry, M. and L. Debarbieux (2012). "Tools from viruses: bacteriophage successes and beyond." Virology **434**(2): 151-161.

Hong, Y., et al. (2014). "MEAT SCIENCE AND MUSCLE BIOLOGY SYMPOSIUM: Development of bacteriophage treatments to reduce O157: H7 contamination of beef products and produce." Journal of animal science **92**(4): 1366-1377.

Hudson, J. A., et al. (2010). "Application of bacteriophages to control pathogenic and spoilage bacteria in food processing and distribution." Bacteriophages in the control of food- and waterborne pathogens: 119-135.

Hussein, H. and T. Sakuma (2005). "Invited review: prevalence of Shiga toxin-producing Escherichia coli in dairy cattle and their products." Journal of Dairy Science **88**(2): 450-465.

Hyman, P. and S. T. Abedon (2009). Practical methods for determining phage growth parameters. Bacteriophages, Springer**:** 175-202.

Jassim, S., et al. (2012). "Novel phage-based bio-processing of pathogenic Escherichia coli and its biofilms." World Journal of Microbiology and Biotechnology **28**(1): 47-60.

Jessup, C. M. and S. E. Forde (2008). "Ecology and evolution in microbial systems: the generation and maintenance of diversity in phage–host interactions." Research in Microbiology **159**(5): 382-389.

Keen, E. C. (2012). "Felix d'Herelle and our microbial future." Future microbiology(7): 1337-1339.

Kim, J.-W., et al. (2008). "Host ranges of Listeria-specific bacteriophages from the turkey processing plant environment in the United States." Applied and environmental microbiology **74**(21): 6623-6630.

King, A. M., et al. (2011). Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses, Elsevier.

Kropinski, A. M., et al. (2009). Enumeration of bacteriophages by double agar overlay plaque assay. Bacteriophages, Springer**:** 69-76.

Kudva, I. T., et al. (1999). "Biocontrol of Escherichia coli O157 with O157-specific bacteriophages." Applied and environmental microbiology **65**(9): 3767-3773.

Kumar, C. G. and S. Anand (1998). "Significance of microbial biofilms in food industry: a review." International journal of food microbiology **42**(1): 9-27.

Labrie, S. J. and S. Moineau (2007). "Abortive infection mechanisms and prophage sequences significantly influence the genetic makeup of emerging lytic lactococcal phages." Journal of bacteriology **189**(4): 1482-1487.

Labrie, S. J., et al. (2010). "Bacteriophage resistance mechanisms." Nature Reviews Microbiology **8**(5): 317-327.

Lake, R., et al. (2000). "Estimated number of cases of foodborne infectious disease in New Zealand." The New Zealand Medical Journal **113**(1113): 278-281.

Lake, R. J., et al. (2010). "Risk ranking for foodborne microbial hazards in New Zealand: burden of disease estimates." Risk Analysis **30**(5): 743-752.

Loc-Carrillo, C. and S. T. Abedon (2011). "Pros and cons of phage therapy." Bacteriophage **1**(2): 111-114.

Low, J. and W. Donachie (1997). "A review of Listeria monocytogenes and listeriosis." The Veterinary Journal **153**(1): 9-29.

Lu, Z. and F. Breidt (2015). "Escherichia coli O157: H7 bacteriophage Φ241 isolated from an industrial cucumber fermentation at high acidity and salinity." Frontiers in microbiology **6**.

Lwoff, A. (1953). "Lysogeny." Bacteriological reviews **17**(4): 269.

Mahony, J., et al. (2011). "Bacteriophages as biocontrol agents of food pathogens." Current Opinion in Biotechnology **22**(2): 157-163.

Marsh, M. and A. Helenius (2006). "Virus entry: open sesame." Cell **124**(4): 729-740.

Mathusa, E. C., et al. (2010). "Non-O157 Shiga toxin–producing Escherichia coli in foods." Journal of Food Protection® **73**(9): 1721-1736.

Matsuzaki, S., et al. (2005). "Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases." Journal of infection and chemotherapy **11**(5): 211-219.

Maura, D. and L. Debarbieux (2011). "Bacteriophages as twenty-first century antibacterial tools for food and medicine." Applied Microbiology and Biotechnology **90**(3): 851-859.

Meredith, L., et al. (2001). "Contributory factors to the spread of contamination in a model kitchen." British Food Journal **103**(1): 23-36.

Monk, A., et al. (2010). "Bacteriophage applications: where are we now?" Letters in applied microbiology **51**(4): 363-369.

Montañez-Izquierdo, V. Y., et al. (2012). "Use of epifluorescence microscopy to assess the effectiveness of phage P100 in controlling Listeria monocytogenes biofilms on stainless steel surfaces." Food Control **23**(2): 470-477.

Newell, D. G., et al. (2010). "Food-borne diseases—the challenges of 20years ago still persist while new ones continue to emerge." International journal of food microbiology **139**: S3-S15.

Nyachuba, D. G. (2010). "Foodborne illness: is it on the rise?" Nutrition Reviews **68**(5): 257-269.

Olaimat, A. N. and R. A. Holley (2012). "Factors influencing the microbial safety of fresh produce: a review." Food Microbiology **32**(1): 1-19.

Ozer, N. P. and A. Demirci (2006). "Inactivation of Escherichia coli O157: H7 and Listeria monocytogenes inoculated on raw salmon fillets by pulsed UV-light treatment." International journal of food science & technology **41**(4): 354-360.

Parashar, U., et al. (2001). "" Norwalk-like viruses". Public health consequences and outbreak management." MMWR. Recommendations and reports: Morbidity and mortality weekly report. Recommendations and reports/Centers for Disease Control **50**(RR-9): 1-17.

Patterson, M. (2005). "Microbiology of pressure-treated foods." Journal of applied microbiology **98**(6): 1400-1409.

Puck, T. T., et al. (1951). "The mechanism of virus attachment to host cells I. The role of ions in the primary reaction." The Journal of experimental medicine **93**(1): 65-88.

Rakhuba, D., et al. (2010). "Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell." Pol. J. Microbiol **59**(3): 145-155.

Ramaswamy, V., et al. (2007). "Listeria-review of epidemiology and pathogenesis." Journal of Microbiology Immunology and Infection **40**(1): 4.

Ramos, B., et al. (2013). "Fresh fruits and vegetables—an overview on applied methodologies to improve its quality and safety." Innovative Food Science & Emerging Technologies **20**: 1-15.

Rice, K. C. and K. W. Bayles (2008). "Molecular control of bacterial death and lysis." Microbiology and Molecular Biology Reviews **72**(1): 85-109.

Ripp, S. and R. V. Miller (1998). "Dynamics of the pseudolysogenic response in slowly growing cells of Pseudomonas aeruginosa." Microbiology **144**(8): 2225-2232.

Rivas, L., et al. (2007). "Attachment of Shiga toxigenic Escherichia coli to stainless steel." International journal of food microbiology **115**(1): 89-94.

Roberts, A. and M. Wiedmann (2003). "Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis." Cellular and Molecular Life Sciences CMLS **60**(5): 904-918.

Rocourt, J., et al. (2003). "The present state of foodborne disease in OECD countries." Geneva: WHO **1**.

Roux, S., et al. (2015). "Viral dark matter and virus–host interactions resolved from publicly available microbial genomes." eLife **4**: e08490.

Sano, E., et al. (2004). "Movement of viruses between biomes." Applied and environmental microbiology **70**(10): 5842-5846.

Schlech, W. F. and D. Acheson (2000). "Foodborne listeriosis." Clinical Infectious Diseases **31**(3): 770-775.

Shafia, F. and T. Thompson (1964). "Calcium ion requirement for proliferation of bacteriophage φμ-4." Journal of bacteriology **88**(2): 293-296.

Shao, Y. and N. Wang (2008). "Bacteriophage adsorption rate and optimal lysis time." Genetics **180**(1): 471-482.

Sharp, R. (2001). "Bacteriophages: biology and history." Journal of Chemical Technology and Biotechnology **76**(7): 667-672.

Sillankorva, S. M., et al. (2012). "Bacteriophages and their role in food safety." International journal of microbiology **2012**.

Smelt, J. (1998). "Recent advances in the microbiology of high pressure processing." Trends in Food Science & Technology **9**(4): 152-158.

Strauch, E., et al. (2007). "Bacteriophages: new tools for safer food?" Journal für Verbraucherschutz und Lebensmittelsicherheit **2**(2): 138-143.

Sulakvelidze, A., et al. (2001). "Bacteriophage therapy." Antimicrobial agents and chemotherapy **45**(3): 649-659.

Summers, W. C. (2001). "Bacteriophage therapy." Annual Reviews in Microbiology **55**(1): 437-451.

Taege, A. (2004). "Foodborne disease." The Cleveland Clinic Disease Management Project, The Cleveland Clinic Foundation, Cleveland OH.

Tauxe, R. V. (1997). "Emerging foodborne diseases: an evolving public health challenge." Emerging infectious diseases **3**(4): 425.

Tauxe, R. V., et al. (2010). "Evolving public health approaches to the global challenge of foodborne infections." International journal of food microbiology **139**: S16-S28.

Van Twest, R. and A. M. Kropinski (2009). "Bacteriophage enrichment from water and soil." Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions: 15-21.

Vongkamjan, K., et al. (2012). "Silage collected from dairy farms harbors an abundance of listeriaphages with considerable host range and genome size diversity." Applied and environmental microbiology **78**(24): 8666-8675.

Vos, T., et al. (2013). "Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010." The Lancet **380**(9859): 2163-2196.

Warriner, K. and A. Namvar (2009). "What is the hysteria with Listeria?" Trends in Food Science & Technology **20**(6): 245-254.

WHO, F. S. U., 1998. Food safety issues: Surface decontamination of fruits and vegetables eaten raw: a review. Geneva: World Health Organization (1998). Food Safety Unit, 1998. Food safety issues: Surface decontamination of fruits and vegetables eaten raw: a review. Geneva: World Health Organization, WHO/FSF/FOS/98.2. V o lu me 35 Nu.

Wouters, P. C., et al. (2001). "Critical factors determining inactivation kinetics by pulsed electric field food processing." Trends in Food Science & Technology **12**(3): 112-121.

Yaun, B. R., et al. (2004). "Inhibition of pathogens on fresh produce by ultraviolet energy." International journal of food microbiology **90**(1): 1-8.

Chapter 2: Bacteriophage Isolation and Characterisation

2.0 Abstract

Increasing globalisation and continuing advances in technology have altered the nature of foodborne disease which have developed from local and contained cases, to widespread global outbreaks. This change has prompted research into alternative methods for pathogen control to mitigate outbreaks of prevalent foodborne pathogens, including *Listeria monocytogenes* and *Escherichia coli*. One such alternative is the use of bacteriophages (phages) as a biocontrol agent. In this chapter, phages were analysed using transmission electron microscopy, host range analysis and adsorption assays. In total five phages were characterised, two *Listeria* phages and three *E. coli* phages, two of which were isolated from faecal matter. The *Listeria* phage CTLLm3 was identified to be a member of the *Siphoviridae* family, with a broad host range and was found to be fast adsorbing. The two *E. coli* phages CJNEc1 and CJNEc2 are members of the *Myoviridae* family, the other isolate AAPEc6 is a member of the *Podoviridae* family. All three phages were found to have a limited host range and fast adsorbing.

2.1 Introduction

Over the past decade the previously contained and isolated nature of foodborne disease has advanced into more widespread, global, outbreaks, resulting in an increase in the number of reported cases worldwide (Hall, Vally et al. 2008). This global rise is thought to be due to continuing globalisation and advancements in technology which have resulted in the increased movement of foods and ingredients, thus pathogens across the world (Altekruse, Cohen et al. 1997). Foodborne diseases are defined as a group of illnesses caused following consumption of contaminated food or water (Tauxe, Doyle et al. 2010). Severity of the illness can range from mild sickness to death and is often dependent on factors including immune system functioning and age, with vulnerable members of the population, such as the elderly, young children and immune compromised individuals being more susceptible to infection (Rocourt, Moy et al. 2003). Although there are many foodborne diseases, among the most prevalent are those attributed to *Listeria monocytogenes* and *Escherichia coli* infections (Rocourt, Moy et al. 2003).

L. monocytogenes was first isolated in 1926 from rabbits and guinea-pigs, and has since been isolated from other sources including plants, soil, surface water, silage and faecal matter (Farber and Peterkin 1991). Research has shown that the pathogen has the ability to survive and grow at a range of temperatures including under refrigeration temperatures, pHs, salt conditions and within biofilms (Roberts and Wiedmann 2003). These characteristics contribute to the ability of the pathogen to grow on food processing surfaces, floors, drains, on equipment and in refrigerated rooms (Carpentier and Cerf 2011). Such growth can often result in contamination of food products, and if consumed in sufficient concentrations can lead to the development of the foodborne disease listeriosis (Ramaswamy, Cresence et al. 2007). Contraction of listeriosis is relatively rare and causes symptoms associated with gastroenteritis, however these cases are generally not fatal (Warriner and Namvar 2009). Despite this, contraction of the invasive systemic disease can be fatal, as the pathogen moves into the blood stream and spreads through the central nervous system often resulting in the development of fatal conditions such as meningitis and encephalitis (Schlech and Acheson 2000). The infection has a high fatality rate of between 20-30% in humans (Newell, Koopmans et al. 2010).

Like *L. monocytogenes*, *E. coli* is considered a serious foodborne pathogen and has been implicated in outbreaks associated with a variety of foods including ice-cream, fruits and more commonly deli salads and meats, among others (Barlow, Gobius et al. 2006). The pathogen ranges in size from 1-3 µm and has been isolated from a variety of foods as it has

the ability to survive and grow in a range of conditions, including biofilms, temperatures between 10°C and 46°C and in acidic environments (Farrokh, Jordan et al. 2013). Combined, these factors increase the potential for contamination of preparation surfaces and food products, making *E. coli* a serious risk factor in the food industry (Farrokh, Jordan et al. 2013). Human consumption of *E. coli* can result in symptoms generally associated with foodborne disease, including vomiting and diarrhoea, although these conditions are generally not fatal (Fairbrother and Nadeau 2006). However, severe infection can result in the development of potentially fatal conditions including acute or chronic renal failure, strokes and nervous system abnormalities (Hussein and Sakuma 2005).

The resistant and adaptable nature of foodborne pathogens, including *L. monocytogenes* and *E. coli* has resulted in the implementation of a range of control measures aimed at preventing contamination of foods and their preparation surfaces. Such controls include the use of chemical sanitizers, UV irradiation, pasteurisation and high pressure processing among others (Ramos, Miller et al. 2013). Despite these efforts incidences of food related illness continue to increase worldwide, prompting the investigation of alternative control measures (Hagens and Loessner 2007). One such alternative is the use of phages as a biocontrol tool (Hagens and Loessner 2007). There are a number of advantages associated with phage biocontrol. Firstly, phages are naturally occurring, host specific infectious agents of bacteria and have bacteriocidal effects (Garcia, Martinez et al. 2008). Furthermore, they can be applied from the beginning to the end of food production systems, can be used as a decontamination tool for carcasses, fruits and vegetables and their widespread applications used individually and in combination with other methods, has the potential to significantly reduce bacterial contamination and therefore, associated illness (Garcia, Martinez et al. 2008). Despite the many advantages associated with phage biocontrol there are limitations which have to be addressed, including the potential for the development of host resistance and lysogenic conversion (Hagens and Loessner 2007). To understand and overcome these limitations, research into the nature of phages and their interactions with bacterial hosts must continue. Therefore, the aim of the research described in this chapter was to isolate *Listeria* and *E. coli* phages from faecal matter, dairy swabs and wastewater samples. Secondly, to characterise these together with previously isolated phages in our collection using transmission electron microscopy, host range analysis and adsorption assays.

2.2 Materials and Methods

2.2.1 Reference cultures and phage stocks

2.2.1.1 Bacterial cultures

The bacterial cultures used for *Listeria* phage testing were obtained from the Christchurch Science Centre, ESR culture collection. The *L. monocytogenes* cultures used were NZRM 2000/47, NZRM 3009, NZRM 3449, NZRM 3450, NZRM 2592, NZRM 2595, NZRM 3370, NZRM 44, NZRM 2594, NZRM 2597, NZRM 3371 and *Listeria ivanovii* NZRM 797. The *E. coli* cultures used were NZRM 1421, NZRM 1403, NZRM 1345, NZRM 3614 and NZRM 4155, NZRM 104329, ERL 063148, ERL 050583, 14ER0097, 14RL0098, ERL034525, 8023, 032832, 056545, 102360, ERL090747, ERL 103290 and ERL071595.

2.2.1.2 Phage cultures

The *Listeria* phages CTLLm3 and AAPEc6 were obtained from the Christchurch Science Centre, ESR. CTLLm3 was isolated by ESR from cheese and A511 was originally isolated from sewage (Loessner and Busse, 1990). The *E. coli* phage AAPEc6 was from the ESR phage culture collection and was isolated from wastewater.

2.2.2 Media

2.2.2.1 E. coli media

Lennox L base (LB) medium (Appendix A2) was used for the agar layer method (Adams, 1959), which comprised LB broth for preparation of overnight and 3-4 hour exponential phase indicator suspensions, LB overlays (Appendix A.2.3) and LB agar (Appendix A.2.2) as base plates for culturing *E. coli*. All *E. coli* media were supplemented with 10mM $MgSO_4$.

2.2.2.2 Listeria media

Tryptic soy base medium (Appendix A3) was used for the soft agar overlay method (Adams, 1959), this comprised TSB broth (Appendix A.3.1), for preparation of overnight and 3-4 hour exponential phase indicator host culture suspensions, TSB overlays (Appendix A.3.3) and TSB agar (Appendix A.3.2) for base plates and culturing of *Listeria*. All *Listeria* media were supplemented with 1.25mM $CaCl_2$.

2.2.3 Agar layer method (soft overlay method)

Agar base plates were poured deeply with 20-30ml of medium, with a required agar concentration of 1-1.5% (Adams, 1959). Soft agar overlays required an agar concentration of 0.6-0.7% (Adams, 1959). Soft overlay agar was prepared earlier and stored in 3-4ml quantities and melted when required, by autoclave steaming at 100˚C for 15 minutes, or by boiling and tempering in a water bath set to 48.9˚C prior to use. Before pouring overlays onto base plates, 0.1 ml exponential phase indicator culture and 0.1ml phage were added, vortex mixed and poured onto previously set and dried base plates. The soft overlay agar was swirled in base plates to ensure consistent covering and left to dry for 15 minutes before inverting and incubating at the optimum host temperature. Following incubation plates were examined for plaques.

2.2.4 Preparing a Listeria bacterial lawn in soft-agar overlay

Listeria indicator hosts were streaked onto TSA agar plates and incubated at 30˚C for 24 hours to grow single colonies. 10ml of TSA + Ca broth was then inoculated with a single colony, and the culture was incubated at 30˚C for 18-24 hours. 10ml of TSA + Ca broth was then inoculated with 0.1 ml of the overnight culture and incubated at 30˚C for 3-4 hours to achieve an exponential culture, and a bacterial lawn sufficient for observing plaques. 0.1 ml of the 3-4 hour subculture, 0.1ml phage stock and 0.2 ml $CaCl_2$ was then added to the soft overlay, vortex mixed and swirled onto the base plate.

2.2.5 Preparing a E. coli bacterial lawn in soft-agar overlay

E. coli indicator hosts were streaked onto LB agar plates and incubated at 37˚C for 18-24 hours to grow single colonies. 10 ml of LB broth was inoculated with a single colony, and the culture was incubated at 37˚C for 18-24 hours. 10ml of fresh LB broth was then inoculated with 0.1ml of the overnight culture and placed back in the 37˚C incubator for 3-4 hours to achieve an exponential host culture and a bacterial lawn sufficient for plaque visualisation. Following this, 0.1ml of the 3-4 hour subculture and 0.1ml of phage stock was added to the soft overlay, vortex mixed and swirled onto base plates.

2.2.6 Spot Plate Technique

0.1ml of the exponential phase host culture was added to prepared overlay tubes. Tubes were vortexed and poured onto previously dried base plates that were labelled in a grid format to allow for later identification of samples. Plates were then left to dry in the biohazard hood for at least 15 minutes. Once plates were set and dried 10µl of serially

diluted phage stock was spotted across the plate, before further drying for at least 15 minutes in the biohazard hood, inverting and incubating at optimum host conditions. For *E. coli* phages the optimal incubation temperature is 37°C and for *Listeria* phages the optimal incubation temperature is 30°C. Plates were observed for plaques 18-24 hours after incubation.

2.2.7 Bacteriophage isolation

The reference cultures used for *Listeria* and *E. coli* phages are as listed in section 2.2.1.1.

2.2.7.1 Isolation of Listeria phage from faecal matter

10g of faecal matter was weighed into medium sized Whirlpak bags, 20 ml of TSB + Ca was added to create a 1:2 dilution, and the mixture was placed in the stomacher machine and left for 30 seconds.

Following this, 0.1ml of host indicator culture was added and the enrichment was incubated for 48 hours at 25˚C. After the 48 hour incubation period, liquid was transferred into a centrifuge tube and centrifuged at 4000g for 15 minutes. The supernatant was transferred into another tube and the pellet discarded. The supernatant was then passed through a 0.22µm syringe filter and stored at 4˚C. 0.1ml of phage filtrate was analysed using the soft agar overlay method on TSAYE base plates and overlays (as in 2.2.3 and 2.2.4). Plates were dried and incubated at 25˚C for 48 hours. At 24 and 48 hours plates were observed for plaques. The host reference cultures used were *L. monocytogenes* NZRM 2000/47, NZRM 3009 and NZRM 3449.

2.2.7.2 Isolation of E. coli phage from faecal matter

The same protocol as 2.2.7.1 was used for the isolation of *E. coli* phages. However, *E. coli* specific media was used. Incubation of the enrichment and overlaid plates was at 37˚C for 18 - 24 hours. Reference cultures used were NZRM 1421, NZRM 1403, NZRM 1326 and NZRM 3641.

2.2.7.3 Isolation of E. coli phages from dairy plant wastewater samples

Wastewater samples were supplied by a local dairy company, samples were received once every two weeks for a period of six weeks. All samples were analysed using enrichment and direct plating methods, as described below.

For the direct plating method samples were centrifuged at 3000 g for 10 minutes and the supernatant filtered through a 0.22 µm filter. Samples were analysed using the overlay method as described above and plates were incubated at 37˚C for 18-24 hours for *E. coli* phages and 24 hours at 30˚C for *Listeria* phages. Plaque formation was checked after incubation.

For the enrichment method, samples were centrifuged at 3000 g for 10 minutes and supernatant filtered through a 0.22 µM filter. 50 ml of the enrichment samples were mixed with an equal volume of dsLB and further divided into 10 ml batches. Following this, 0.5 ml of the log phase host was added and samples were incubated at 37˚C for 5-6 hours in the case of *E. coli* phages, or 30˚C for 6-7 hours in the case of *Listeria* phages. Following the incubation period samples were centrifuged at 3000 g for 10 minutes and the supernatant filtered through a 0.22 µm filter. Samples were analysed using the spot plate method (as described in 2.2.6), before plates were incubated at 37˚C for 18-24 hours for *E. coli* phages and 30˚C for 6-7 hours for *Listeria* phages

2.2.8 Characterisation experiments

2.2.8.1 Host range determination

For host range studies the spotting technique was used as described in 2.2.6. High titre phage stocks were serially diluted by mixing 0.9 ml of SM buffer with 0.1 ml phage stock. The original host culture and other possible indicator cultures were prepared as described above in sections 2.2.4 and 2.2.5. Each overlay was then inoculated with 0.1 ml of *Listeria* or *E. coli* host suspension and poured onto a previously dried base plate, which was labelled in a grid format to allow identification of each dilution. Once plates were dry 10 µl spots of each diluted phage were spotted across the overlay. Plates were dried and incubated at optimum host conditions, and analysed for plaques.

2.2.8.2 Host range quantification of phage

For host range quantification, the overlay method was used. Exponential phase cultures of the original indicator bacteria and test host strains were prepared as described in sections 2.2.4 and 2.2.5. High titre phage stocks were serially diluted as mentioned above. Each overlay was inoculated with 0.2 ml of exponential bacterial culture and 0.1 ml of the serially diluted phage stock. Plates were then incubated under optimum growth conditions of the

host. After the incubation period plaques were enumerated and the dilution factor considered.

2.2.9 Preparation for transmission electron microscopy (TEM)

2.2.9.1 Preparing fresh high titre stocks

For electron microscopy studies concentrated phage stocks are required. Therefore, fresh high titre stocks were prepared by plating 10-20 plates of the current phage stock using the overlay method to obtain plates giving confluent lysis. Plates were incubated overnight under optimum host conditions. After incubation 5 ml SM buffer was added to each plate and swirled at room temperature for 4 hours. The liquid and soft agar was then scraped into disposable centrifuge tubes and centrifuged at 3000 g for 15 minutes. The liquid was filtered through a 0.22 µm filter into a non-disposable sterile centrifuge tube and centrifuged at maximum G-force overnight. The supernatant was carefully removed and 0.1M ammonium acetate added and mixed gently before centrifuging again at G-force overnight. This wash step was repeated, thus there was a total of three washes. After the last wash step the supernatant was gently removed and the pellet re-suspended in 1 ml of 0.1M ammonium acetate and mixed gently. This mixture was transferred into a sterile bijou tube, covered and packed into a travel container with ice. The titre of the stocks is preferably $>10^{11}$ pfu/ml to allow for visualisation.

Sensitive phages were prepared using a liquid infection method, 5 ml of appropriate broth was inoculated with a host colony and incubated at the optimum host temperature overnight. Following this, 50 ml of fresh broth was inoculated with 0.5ml of the overnight culture, and grown with rapid shaking for 3-4 hours at the optimum host temperature. 10^9 pfu/ml of phage was then added to the grown bacterial culture before leaving for 3-4 hours or until lysis was observed. The mixture was then transferred into a centrifuge tube and centrifuged at 4000 g for 10 minutes to pellet the cells. The liquid was then filtered through a 0.22 µm filter and an aliquot taken for enumeration using the standard overlay method. The filtered sample was further prepared using the above washing steps with ammonium acetate.

2.2.9.2 Visualisation of phages using transmission electron microscope

Phage morphologies were visualised using a transmission electron microscope with a negative staining method undertaken at the University of Otago. The method involved the

application of a drop of freshly prepared phage stock to a 200-mesh copper grid, which was left to dry for 1 to 2 minutes. Meanwhile, excess liquid is removed using filter paper. A drop of 2% aqueous phosphotungstic acid, buffered to pH 6.5 was then applied for 15 to 25 seconds. The excess liquid was then removed with filter paper and the grid was left to air dry, before mounting and visualising at 100,000-260,000 x magnification.

2.2.10 Adsorption assay

For *E. coli* adsorption assays, LB broth supplemented with 10mM $MgSO_4$ was used, whereas for *Listeria* experiments TSB supplemented with 1.25mM $CaCl_2$ was used.

Host cells were prepared to the exponential mid-log phase as described in sections 2.2.4 and 2.2.5. 10 ml of the relevant broth was dispensed into tubes and 0.1 ml of exponential host cells diluted to 4 x 10^6 cells/ml and 0.1 ml of phage diluted to 5 x 10^3 pfu/ml were added and vortex mixed. Tubes were incubated in a shaking water bath at 37˚C for *E. coli* and 30˚C for *Listeria*. 0.8 ml aliquots were taken at 0, 4, 8, 12, 16, 20, 24 and 28 minutes, immediately filtered through a 0.22µm filter and directly plated using the overlay method (Shao & Wang, 2008). The experiment was repeated three times, or until consistent results were obtained.

The k value of each of the phages was calculated using the equation by (Hyman and Abedon 2009).

$$\ln P = -(kN)*t + \ln P_0$$

Where N is bacterial density (CFU/ml), P_0 is the initial un-adsorbed phage titre, P is the final un-adsorbed phage titre both measured in pfu/ml, k is the adsorption rate constant (ml/min) and t is the phage exposure time (min). The k value for each of the phages was calculated from three replicate experiments.

2.3 Results

2.3.1 Bacteriophage isolation

2.3.1.1 Listeria phage isolation

To isolate new *Listeria* phages several types of environmental samples were tested for the presence of phages through direct isolation and enrichment methods (Table 2.1). Faecal samples enriched with the hosts NZRM 2000/47, NZRM 3449 and NZRM 3009 yielded plaques through the spot plate method when plated on respective hosts. However, no phages were isolated as plaques were not evident during subsequent purification and propagation processes. The phages CTLLm3, lytic on the host NZRM 3449, and A511, lytic on *L. ivanovii* NZRM 797, were obtained from the laboratory culture collection for further characterisation studies, and as a reference phage respectively.

Table 2.1: Sample types and methods used for *Listeria* phage isolation.

Sample type	Direct isolation	Enrichment	Total number of samples
Faecal matter	0	21	21
Dairy swabs	3	3	3
Wastewater	12	12	12
Total number of samples			**36**

Note: Faecal samples were analysed through enrichment methods only, whereas dairy swabs and wastewater samples were analysed using both direct and enrichment methods.

2.3.1.2 E. coli phage isolation

To isolate *E. coli* phages several environmental samples were tested through methods of direct isolation and enrichment (Table 2.2). Although no phages were isolated from wastewater samples, two *E. coli* phages were isolated from samples of faecal matter enriched with the strains NZRM 3614, NZRM 1421, NZRM 1345 and NZRM 1403. The phages were lytic on the host NZRM 3614 and the two isolates were named CJNEc1 and CJNEc2. An

uncharacterised phage (AAPEc6), lytic on the host NZRM 1345 was sourced from the ESR laboratory collection and also included in further characterisation studies.

Table 2.2: Sample types and methods used for *E. coli* phage isolation

Sample type	Direct isolation	Enrichment	Total number of samples
Faecal matter	0	17	17
Wastewater	12	12	12
Total number of samples			**29**

Note: Faecal samples were analysed through enrichment methods only, whereas wastewater samples were investigated through both direct and enrichment methods.

2.3.2 Characterisation Experiments

2.3.2.1 Listeria Phage Host Range Determination

The results of the host range analysis for the *Listeria* phages A511 and CTLLm3 are summarised in Table 2.3. CTLLm3 showed lytic potential on seven of the twelve hosts tested, producing small, clear plaques on each host, suggesting that CTLLm3 has a relatively broad host range. Whereas, A511 showed lytic potential on all twelve of the hosts tested, indicating that the phage has a broad host range.

Efficiency of plating (EOP) was also investigated for both *Listeria* phages. EOP is defined as the percentage of plaque counts formed on the test host strain against the number of plaques formed on the lawn of the original indicator host (Kutter 2009). EOP was calculated for all hosts that produced clear plaques using the standard overlay method. The highest EOP result obtained for CTLLm3 (1.057) was from the host strain NZRM 3370, whereas the highest EOP result obtained for A511 (1.000) was from the propagating host for the phage *L. ivanovii* NZRM 797. The EOP data gathered for A511 confirms that the phage has significant lytic ability over a broad host range, however lytic ability varies between hosts (Table 2.3).

Table 2.3: Host Range Determination and quantification for *Listeria* phages CTLLm3, host *L. monocytogenes* NZRM 3449 and A511, host *L. ivanovii* NZRM 797.

Indicator Host	**Serogroup**	**Phage isolates Susceptibility/EOP**	
		CTLLm3	**A511**
NZRM 2000/47	-	(-)	(++) 0.002
NZRM 3009	-	(+)	(++) 0.01
NZRM 3449	1/2a	(++) 1.000	(+)
NZRM 3450	1/2a	(+)	(+)
NZRM 2592	2	(+)	(++) 0.02
NZRM 2595	4a	(+)	(++) 0.004
NZRM 3370	4b6	(++) 1.057	(+) 0.100
NZRM 44	1a	(-)	(++) 0.120
NZRM 2594	3a	(-)	(++) 0.022
NZRM 2597	4d	(-)	(++) 0.001
NZRM 3371	1a1	(-)	(++) 0.001
***L. ivanovii* NZRM 797**	5	(+)	(++) 1.000

Efficiency of plating. Starting titre for CTLLm3 3.50 x 10^6 pfu/ml, starting titre for A511 1.0 x 10^9 pfu/ml. (++) = Clear plaques and phage titre, (+) = Lysis using spotting technique only, (-) = No plaques.

2.3.2.2 E. coli phage host range determination

The results of the host range analysis for the *E. coli* phages CJNEc1, CJNEc2 and AAPEc6 are summarised in Table 2.4. CJNEc1 and CJNEc2 were only lytic on one of the twenty *E. coli* hosts tested. When plated on NZRM 3614 both phages produced small, clear plaques, however did not show lytic ability on any other host tested. AAPEc6 produced large, clear, lytic plaques when plated on NZRM 1345 however, did not lyse any of the other hosts tested. These results indicate that all three phages have a narrow host range (Table 2.4).

Table 2.4: Host Range determination and quantification for *E. coli* phages CJNEc1, CJNEc2 and AAPEc6

Indicator Host	Serogroup	Phage isolates Susceptibility		
		CJNEc1	**AAPEc6**	**CJNEc2**
NZRM 3614	O157	(++)	(-)	(++)
NZRM 1403	O103	(-)	(-)	(-)
NZRM 1421	O121	(-)	(-)	(-)
NZRM 1345	O45:H10	(-)	(++)	(-)
NZRM 4451	-	(-)	(-)	(-)
NZRM 104329	O154:H34	(-)	(-)	(-)
NZRM 1326	O26	(-)	(-)	(-)
ERL063148*	O145	(-)	(-)	(-)
ERL050583	O145	(-)	(-)	(-)
14ER0097	O157	(-)	(-)	(-)
14RL0098	O157	(-)	(-)	(-)
ERL034525	O103	(-)	(-)	(-)
ERL103295	O103	(-)	(-)	(-)
8023	O121	(-)	(-)	(-)
03 2832	O121	(-)	(-)	(-)
05 6545	O45	(-)	(-)	(-)
10 2360	O45	(-)	(-)	(-)
ERL090747	O26	(-)	(-)	(-)
ERL103290	O26	(-)	(-)	(-)
ERL071595	O111	(-)	(-)	(-)

Starting titre for CJNEc1 7.30 x 10^7 pfu/ml, starting titre for AAPEc6 9.50 x 10^6 pfu/ml and starting titre CJNEc2 2.0 x 10^6 pfu/ml. (++) = Clear plaques and phage titre, (-) = No plaques, (+) = Lysis using spotting technique.

2.3.4 Transmission electron microscopy (TEM)

2.3.4.1 Listeria phage electron microscopy

The TEM analysis revealed that the *Listeria* phage CTLLm3 was part of the *Siphoviridae* family, order *Caudovirales*. This is primarily due to the presence of an isometric head and a long non-contractile tail (Fig 2.1). CTLLm3 had an average tail length of 245.1nm +/-0.7 (n=21), an average head diameter of 60.41nm +/- 0.2 (n=21), an average head length of 57.91nm +/- 0.2 (n=21) and a base plate length of 14.78nm +/- 0. 2 (n=21). A511 was not studied by TEM as its morphology is known to be that of a Myovirus.

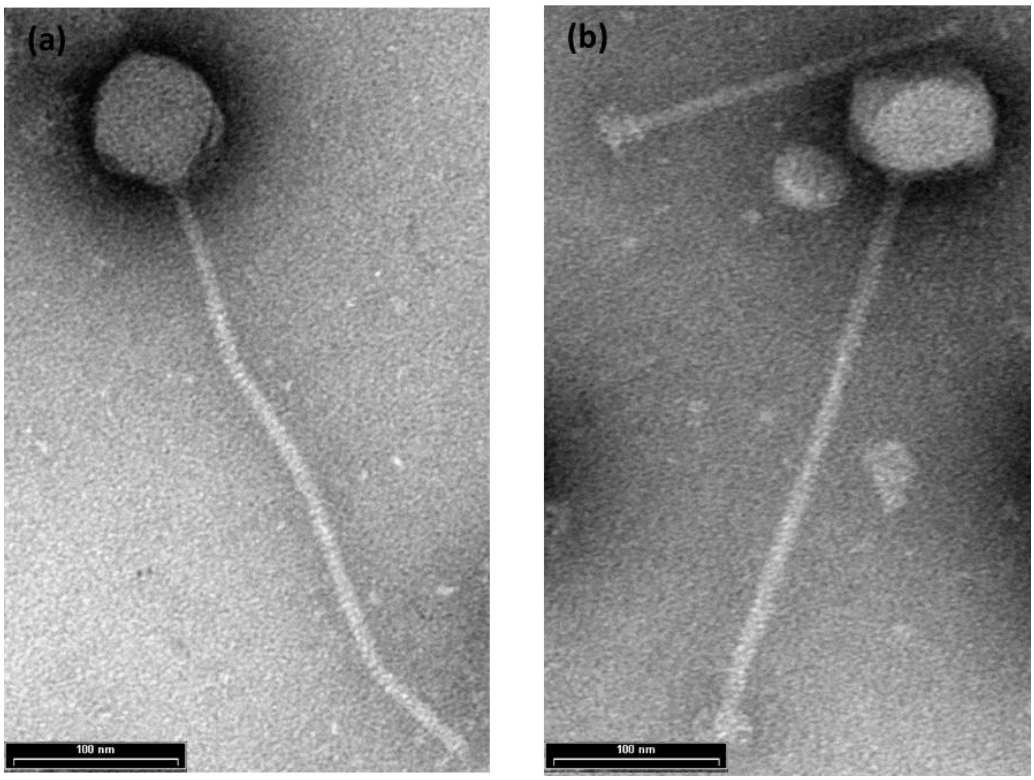

Figure 2.1: (a) and (b) TEM image of CTLLm3, scale bar = 100nm

2.3.4.2 E. coli phage electron microscopy

The *E. coli* phages CJNEc1 and CJNEc2, had similar dimensions and morphology, and were classified as part of the *Myoviridae* family, order *Caudovirales* based on the TEM images (Figure 2.2). Both phages have an isometric head shape and long contractile tails, consistent with other members of this family. CJNEc1 has an average head length of 60nm, an average head diameter of 59.6nm, an average tail length 139.9nm and an average base plate length of 3.57nm. CJNEc2 has an average head length of 61.1nm, an average head diameter of 60.7nm, an average tail length of 144.84nm and an average base plate length of 4.76nm, as shown in Table 2.5.

The TEM images of AAPEc6 indicate that the phage is a member of the *Podoviridae* family, order *Caudovirales* (Figure 2.2). AAPEc6 has an icosahedral head and a small non-contractile tail. AAPEc6 had an average head length of 69.8nm, an average head diameter of 59.6nm, an average tail length of 10.5nm and an average base plate length of 7.05nm, of all the *E. coli* phages AAPEc6 was the smallest, as shown in Table 2.5.

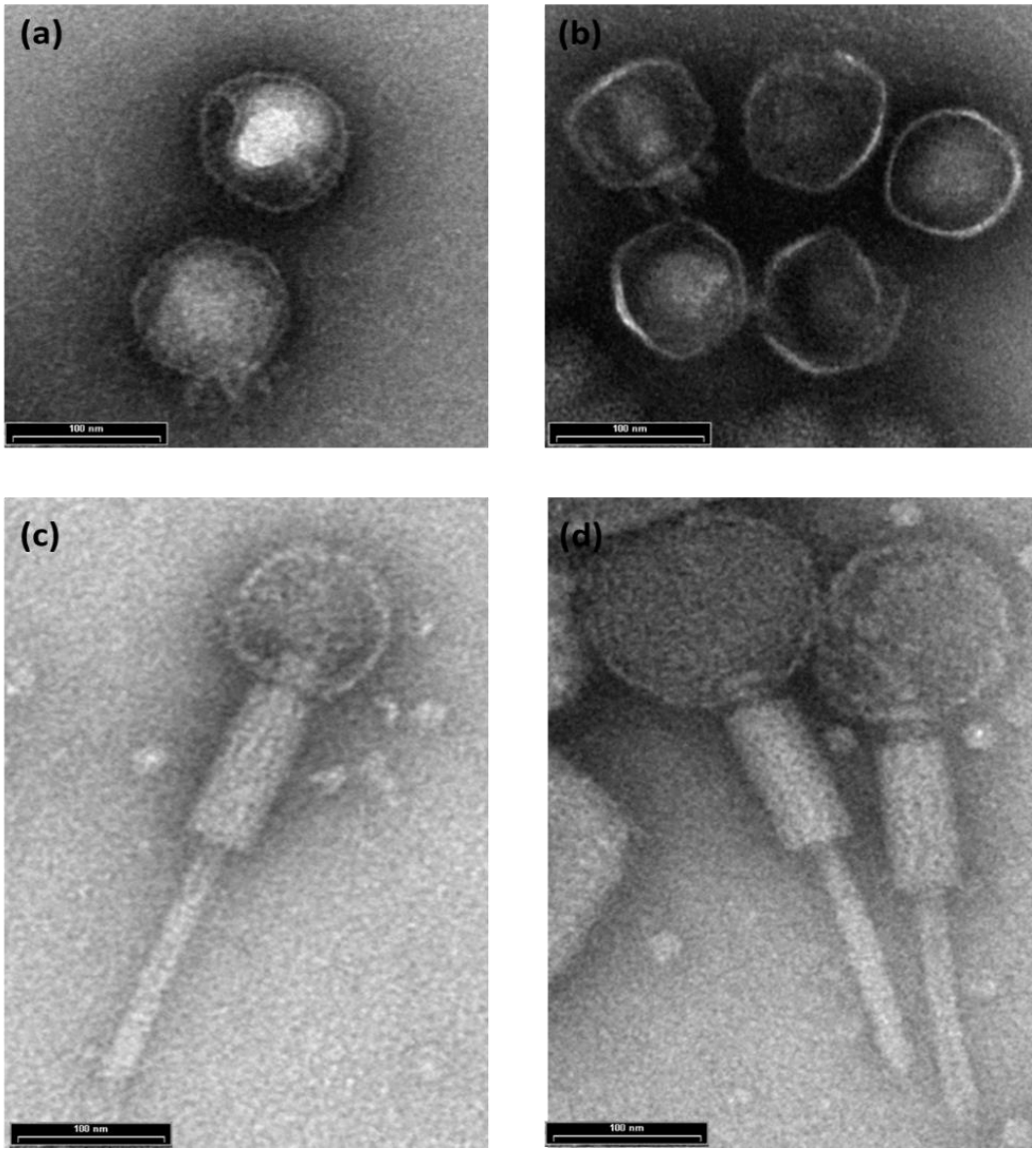

Figure 2.2: (a) and (b) TEM images of *E. coli* phage AAPEc6, (c) TEM image of *E. coli* phage CJNEc1, (d) TEM image of *E. coli* phage CJNEc2. Note that the tail sheath is contracted exposing the tail tube in images of CJNEc1 and CJNEc2. Scale bar = 100nm.

Table 2.5: Comparison of *E. coli* phages AAPEc6, CJNEc1 and CJNEc2 characteristics as determined by TEM analysis

Physical characteristics (nm)	AAPEc6/1345	CJNEc1/3614	CJNEc2/3614
Head length	69.8nm +/- 0.25	60nm +/- 0.25	61.1nm +/- 0.2
Head diameter	59.6nm +/- 0.24	59.6nm +/- 0.30	60.7nm +/- 0.25
Tail length	10.5nm +/- 0.16	139.9nm +/- 1.47	144.84nm +/- 1.6
Base plate length	7.05nm +/- 0.09	3.57nm +/- 0.08	4.76nm +/- 0.04
Number of phage measured	24	26	30
Tail	Non-contractile	Contractile	Contractile
Head Type	Icosahedral	Isometric	Isometric

2.3.5 Adsorption Assays

The ability of phages to attach to surface receptors of their host is the crucial first step in phage replication. Therefore, adsorption assays were completed by adding diluted phages to exponentially prepared bacterial host cultures and plating every 4 minutes over a 28 minute period. Adsorption constant values, k were subsequently calculated to establish the efficiency of phages to attach to their hosts.

2.3.5.1 Listeria Phage Adsorption Assays

Adsorption constant values, k for the phages A511 and CTLLm3 were calculated using the equation by Hyman and Abedon (2009) from results of triplicate experiments. As summarised in Table 2.8 the results indicated that the *Listeria* phage A511 had an average adsorption constant of 5.97^{-10} (Figure 2.3; Table 2.6). Whereas, CTLLm3 had an average adsorption constant of 1.41^{-9} (Figure 2.4; Table 2.7).

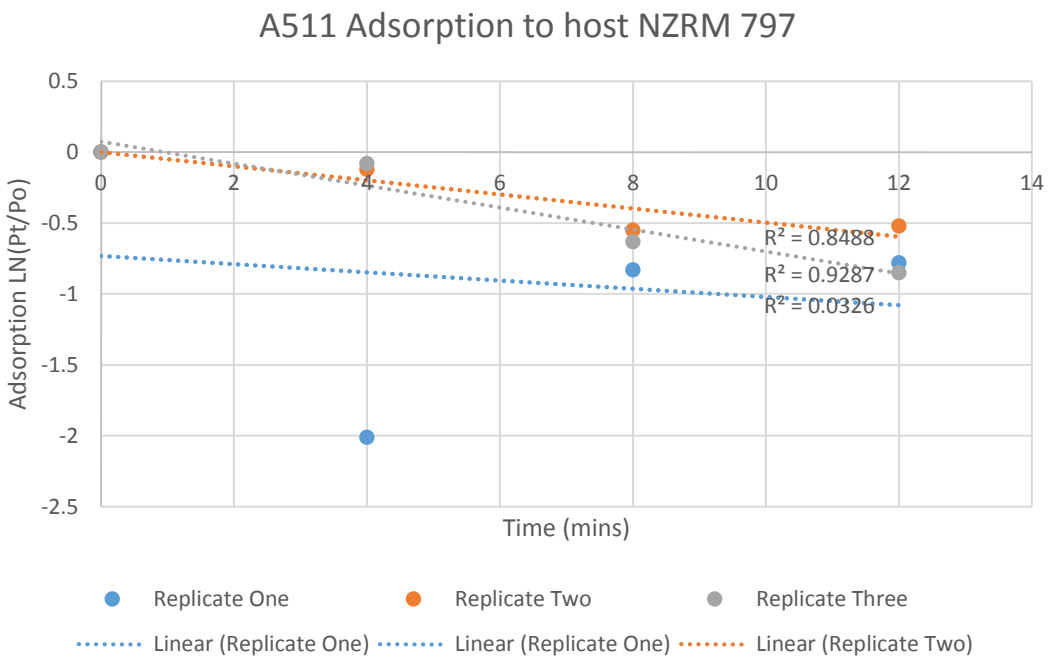

Figure 2.3: Triplicate results of the adsorption kinetics of the *Listeria* phage A511 onto host *L. ivanovii* NZRM 797 over a period of 28 minutes at 30°C.

Table 2.6: Adsorption constant (k) data for A511

A511	k
Replicate One	6.52^{-10}
Replicate Two	4.34^{-10}
Replicate Three	7.05^{-10}
Average k	5.97^{-10}

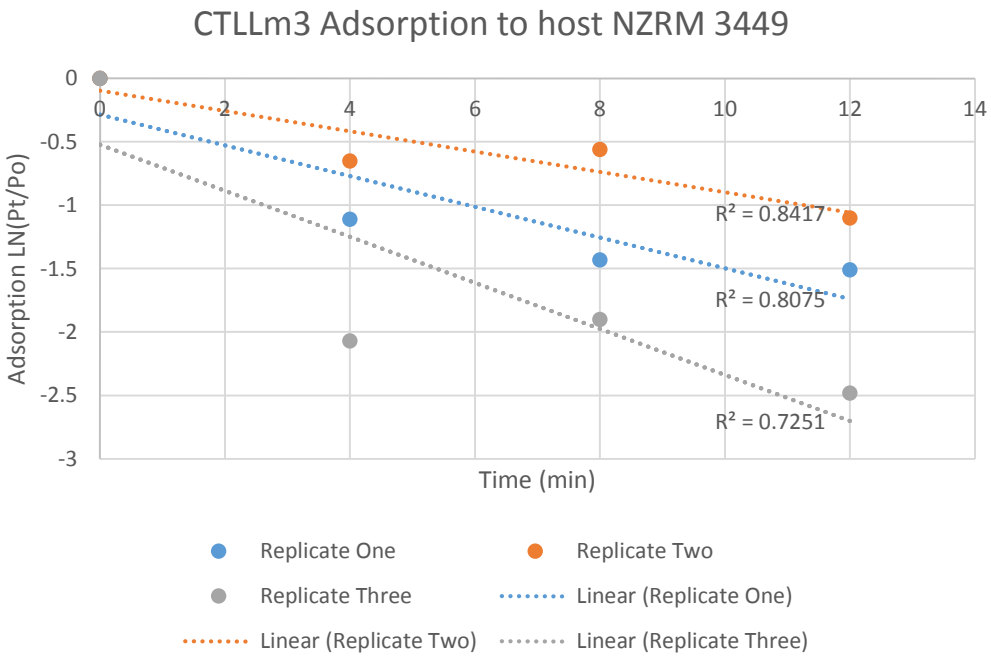

Figure 2.4: Triplicate results of the adsorption kinetics of the *Listeria* phage CTLLm3 onto host *L. monocytogenes* NZRM 3449 over a period of 28 minutes at 37°C.

Table 2.7: Adsorption constant (k) data for CTLLm3

CTLLm3	k
Replicate One	1.26^{-9}
Replicate Two	9.15-^{10}
Replicate Three	2.06^{-9}
Average k	1.41^{-9}

Table 2.8: Summary table of adsorption k values for the *Listeria* phages A511 and CTLLm3

***Listeria* phage**	**Mean k value**
A511	5.97^{-10}
CTLLm3	1.41^{-9}

2.3.5.2 E. coli Phage Adsorption Assays

Analysis of the results from the triplicate experiments indicated that all three *E. coli* phages had adsorption constant values of 10^{-9}. As summarised in Table 2.12, AAPEc6 had an average adsorption constant of 1.49^{-9} (Figure 2.5; Table 2.9), whereas CJNEc1 had an average k value of 2.36^{-9} (Figure 2.6; Table 2.10) and CJNEc2 had a k value of 1.92^{-9} (Figure 2.7; Table 2.11).

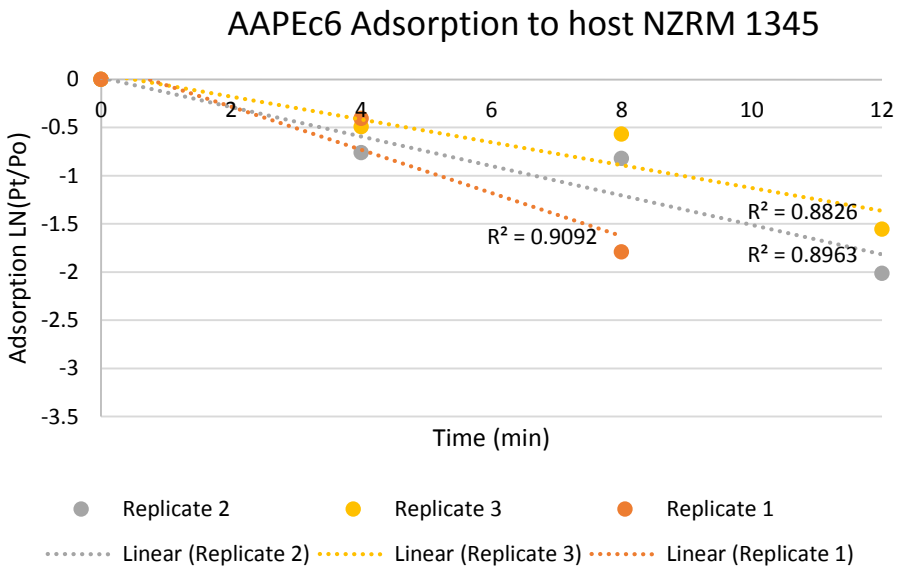

Figure 2.5: Triplicate results of the adsorption kinetics of the *E. coli* phage AAPEc6 onto *E. coli* host NZRM 1345 over a period of 28 minutes at 37°C.

Table 2.9: Adsorption constant (k) data for AAPEc6

AAPEc6	**k**
Replicate One	1.49^{-9}
Replicate Two	1.68^{-9}
Replicate Three	1.30^{-9}
Average k	1.49^{-9}

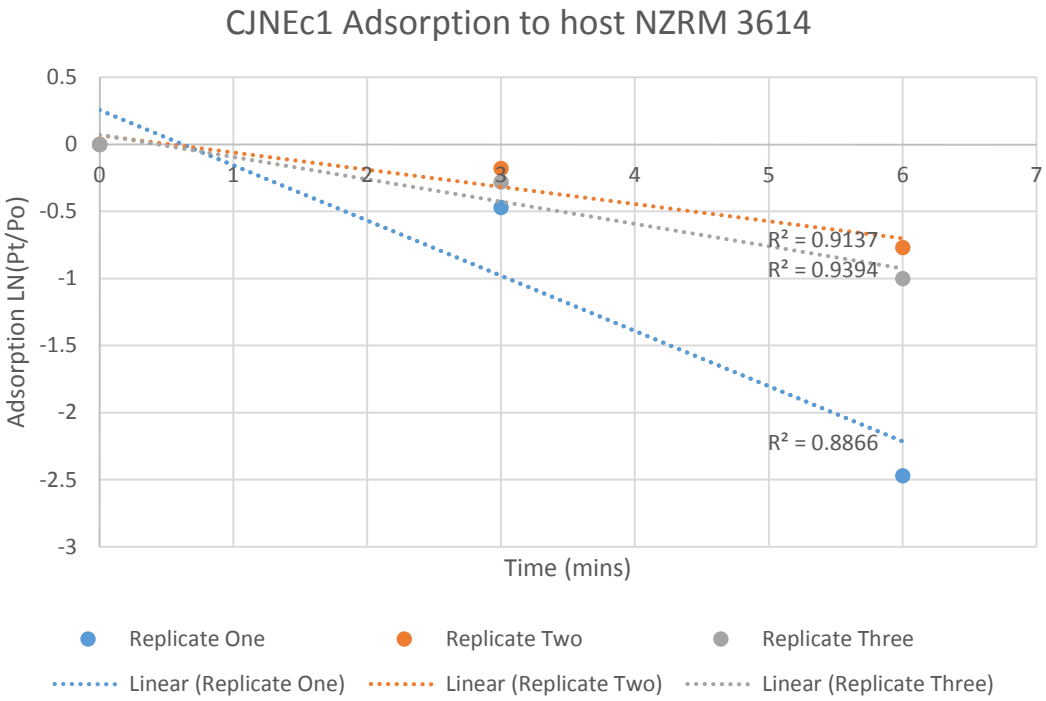

Figure 2.6: Triplicate results of the adsorption kinetics of the *E. coli* phage CJNEc1 onto *E. coli* host NZRM 3614 over a period of 28 minutes at 37°C.

Table 2.10: Adsorption constant (k) data for CJNEc1

CJNEc1	**k**
Replicate One	4.11^{-9}
Replicate Two	1.29^{-9}
Replicate Three	1.67^{-9}
Average k	2.36^{-9}

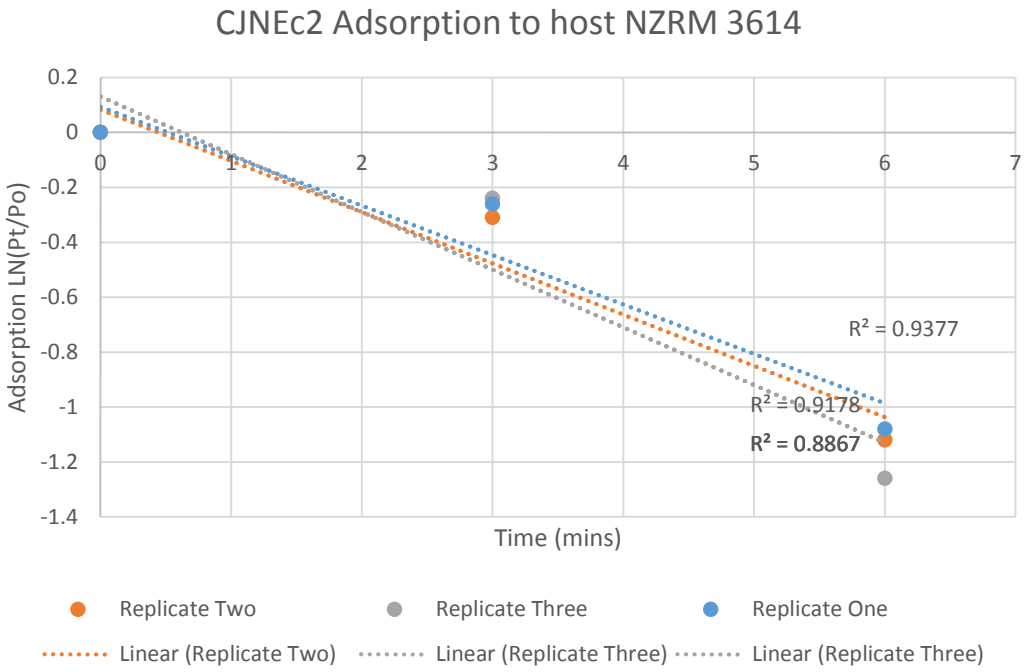

Figure 2.7: Triplicate results of the adsorption kinetics of the *E. coli* phage CJNEc2 onto *E. coli* host NZRM 3614 over a period of 28 minutes at 37°C.

Table 2.11: Adsorption constant (k) data for CJNEc2

CJNEc2	k
Replicate One	1.79479^{-9}
Replicate Two	1.8689^{-9}
Replicate Three	2.09743^{-9}
Average k	1.92037^{-9}

Table 2.12: Summary table of adsorption k values for the *E. coli* phages AAPEc6, CJNEc1 and CJNEc2

***E. coli* phage**	**Mean k value**
AAPEc6	1.49^{-9}
CJNEc1	2.36^{-9}
CJNEc2	1.92^{-9}

2.4 Discussion

2.4.1 Bacteriophage isolation

Two *E. coli* phages lytic on the host NZRM 3614 were isolated from faecal matter, however no phages were isolated from wastewater samples this could be due to chance or experimental design. To increase the possibility of isolating phages from wastewater samples a greater range of *E. coli* host strains could be used during the enrichment and screening stages.

Samples screened for *Listeria* phage isolation included direct and enriched wastewater samples, faecal matter and dairy swabs, although no phages were isolated, previous studies have isolated phages from such samples (Vongkamjan, Switt et al. 2012). It is possible that *Listeria* phages proved harder to isolate than *E. coli* phages due to a comparatively slower growth rate between the organisms and inconsistent growth of lawns at 25°C and 30°C.

2.4.2 Host range experiments

The host range results completed for all five phages suggested that the *E. coli* phages AAPEc6, CJNEc1 and CJNEc2 did not have a broad host range as they showed limited lytic potential on the hosts tested. The limited host range of all three phages suggests that independently they would not be suitable for the purpose of biocontrol, as application is likely to result in rapid resistance to the bacterial host (Hagens and Loessner 2010). Limited host range is likely to contribute to host cell resistance as phages can only infect a specific strain(s) of bacteria (Strauch, Hammerl et al. 2007). Due to this specificity host cells are likely to adapt over time and develop resistance mechanisms that prevent phages from entering, infecting and killing the host cell, thus increasing the frequency of resistant host cells in the population (Hagens and Loessner 2010). For this reason phages with a limited host range are considered less suitable for the purpose of biocontrol in industrial environments (Hagens and Loessner 2010).

Whereas, host range results for the *Listeria* phages CTLLm3 and A511 suggested that both have a relatively broad host range, and therefore could be used for the purpose of biocontrol independently or in a cocktail. The broader host range of A511 indicates that this phage is more suitable as it is less likely to confer resistance to bacterial hosts (Kim, Siletzky et al. 2008). The EOP data gathered confirms that the phage has a broad host range, however, lytic ability varies between hosts.

Phages with a broader host range are considered suitable for industrial application as they are likely to be capable of infecting and killing a wider range of bacterial cells (Mahony, McAuliffe et al. 2011). In environments where host range is unknown phages with the ability to infect a range of host types is advantageous, as they have the potential to eliminate a variety of strains within the population (Hagens and Loessner 2010).

2.4.3 Transmission electron microscopy of phages

The TEM images produced for each of the phages was clear, however, they indicated the presence of contracted tails on the phages CJNEc1 and CJNEc2. Therefore, both phages were re-prepared using a liquid infection method rather than the standard preparation method. However, the resulting images still showed signs of contracted tails on both phages, suggesting that CJNEc1 and CJNEc2 are highly sensitive to the TEM preparation process.

The images obtained confirmed that AAPEc6, which produces the largest plaques was the smallest phage. Whereas, CJNEc1, CJNEc2 and CTLLm3 which produce smaller plaques are larger phages. These results are consistent with studies considering the relationship between phage size and plaque size. Previous research suggests that the larger the phage, the smaller the plaques produced due to the presence of extra appendages (Adams 1959, Gallet, Kannoly et al. 2011). Larger phages are known to have extra appendages, which may limit virion diffusion through the overlay layer of agar, resulting in smaller plaques (Gallet, Kannoly et al. 2011).

2.4.4 Adsorption assay

The first step involved in phage replication is adsorption, the attachment of phage onto the surface receptors of their host (Marsh and Helenius 2006). The adsorption rate constant, k is influenced by factors including temperature, growth medium, surface area and motility of the bacterium where an increase in surface area and motility contribute to a higher value of k (Delbrück 1940). Phages with a higher k value are generally faster at adsorbing and infecting host cells, thus have a shorter optimal lysis time (Shao and Wang 2008). Each of the adsorption experiments in this study was carried out in triplicate, the k values calculated for *E. coli* phages were all at 10^{-9}ml/min. The k values calculated for the *Listeria* phages were between 10^{-9}-10^{-10}ml/min. Phages with k values between 10^{-9}-10^{-10}ml/min are considered fast adsorbing and suitable for biocontrol purposes as they attach and infect surrounding host cells faster than slower adsorbing phages (Shao and Wang 2008, Gallet, Shao et al. 2009). As all the *Listeria* and *E. coli* phages in this study had high k values between 10^{-9}-10^{-}

^{10}ml/min, they are considered fast adsorbing therefore, based on these results would be suitable for biocontrol purposes.

2.4.5 Sequencing data

The characterisation results obtained thus far suggest that the phages AAPEc6 and CTLLm3 show biocontrol potential, therefore the next step in the process of characterisation is obtaining sequence data. Both phages were prepared for sequencing following DNA extraction. However, due to time constraints, results were not obtained in time to be included in this thesis.

2.5 Conclusion

A total of three *E. coli* phages and two *Listeria* phages were characterised by morphology, host range and adsorption studies. The results of these experiments suggested that the *Listeria* phage CTLLm3 and the *E. coli* phage AAPEc6 show biocontrol potential. Therefore, both phages were further investigated using kinetic and applied experiments.

2.6 References:

Adams, M. H. (1959). "Bacteriophages." Bacteriophages.

Altekruse, S., et al. (1997). "Emerging foodborne diseases." Emerging infectious diseases **3**(3): 285.

Barlow, R. S., et al. (2006). "Shiga toxin-producing Escherichia coli in ground beef and lamb cuts: results of a one-year study." International journal of food microbiology **111**(1): 1-5.

Carpentier, B. and O. Cerf (2011). "Review—Persistence of Listeria monocytogenes in food industry equipment and premises." International journal of food microbiology **145**(1): 1-8.

Delbrück, M. (1940). "The growth of bacteriophage and lysis of the host." The journal of general physiology **23**(5): 643.

Fairbrother, J. and E. Nadeau (2006). "Escherichia coli: on-farm contamination of animals." Rev Sci Tech **25**(2): 555-569.

Farber, J. and P. Peterkin (1991). "Listeria monocytogenes, a food-borne pathogen." Microbiological reviews **55**(3): 476.

Farrokh, C., et al. (2013). "Review of Shiga-toxin-producing Escherichia coli (STEC) and their significance in dairy production." International journal of food microbiology **162**(2): 190-212.

Gallet, R., et al. (2011). "Effects of bacteriophage traits on plaque formation." BMC microbiology **11**(1): 181.

Gallet, R., et al. (2009). "High adsorption rate is detrimental to bacteriophage fitness in a biofilm-like environment." BMC evolutionary biology **9**(1): 241.

Garcia, P., et al. (2008). "Bacteriophages and their application in food safety." Letters in applied microbiology **47**(6): 479-485.

Hagens, S. and M. J. Loessner (2007). "Application of bacteriophages for detection and control of foodborne pathogens." Applied Microbiology and Biotechnology **76**(3): 513-519.

Hagens, S. and M. J. Loessner (2010). "Bacteriophage for biocontrol of foodborne pathogens: calculations and considerations." Current pharmaceutical biotechnology **11**(1): 58-68.

Hall, G., et al. (2008). "Foodborne Illnesses: Overview."

Hussein, H. and T. Sakuma (2005). "Invited review: prevalence of Shiga toxin-producing Escherichia coli in dairy cattle and their products." Journal of Dairy Science **88**(2): 450-465.

Hyman, P. and S. T. Abedon (2009). Practical methods for determining phage growth parameters. Bacteriophages, Springer**:** 175-202.

Kim, J.-W., et al. (2008). "Host ranges of Listeria-specific bacteriophages from the turkey processing plant environment in the United States." Applied and environmental microbiology **74**(21): 6623-6630.

Kutter, E. (2009). "Phage host range and efficiency of plating." Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions: 141-149.

Loessner, M. J. and M. Busse (1990). "Bacteriophage typing of Listeria species." Applied and environmental microbiology **56**(6): 1912-1918.

Mahony, J., et al. (2011). "Bacteriophages as biocontrol agents of food pathogens." Current Opinion in Biotechnology **22**(2): 157-163.

Marsh, M. and A. Helenius (2006). "Virus entry: open sesame." Cell **124**(4): 729-740.

Newell, D. G., et al. (2010). "Food-borne diseases—the challenges of 20years ago still persist while new ones continue to emerge." International journal of food microbiology **139**: S3-S15.

Ramaswamy, V., et al. (2007). "Listeria-review of epidemiology and pathogenesis." Journal of Microbiology Immunology and Infection **40**(1): 4.

Ramos, B., et al. (2013). "Fresh fruits and vegetables—an overview on applied methodologies to improve its quality and safety." Innovative Food Science & Emerging Technologies **20**: 1-15.

Roberts, A. and M. Wiedmann (2003). "Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis." Cellular and Molecular Life Sciences CMLS **60**(5): 904-918.

Rocourt, J., et al. (2003). "The present state of foodborne disease in OECD countries." Geneva: WHO **1**.

Schlech, W. F. and D. Acheson (2000). "Foodborne listeriosis." Clinical Infectious Diseases **31**(3): 770-775.

Shao, Y. and N. Wang (2008). "Bacteriophage adsorption rate and optimal lysis time." Genetics **180**(1): 471-482.

Strauch, E., et al. (2007). "Bacteriophages: new tools for safer food?" Journal für Verbraucherschutz und Lebensmittelsicherheit **2**(2): 138-143.

Tauxe, R. V., et al. (2010). "Evolving public health approaches to the global challenge of foodborne infections." International journal of food microbiology **139**: S16-S28.

Vongkamjan, K., et al. (2012). "Silage collected from dairy farms harbors an abundance of listeriaphages with considerable host range and genome size diversity." Applied and environmental microbiology **78**(24): 8666-8675.

Warriner, K. and A. Namvar (2009). "What is the hysteria with Listeria?" Trends in Food Science & Technology **20**(6): 245-254.

Chapter 3: Applied Experiments

3.0 Abstract

The continuing increase in the number reported cases of food related illness and the detection of pathogens on foods and surfaces is driving research into alternative controls. One alternative is the use of phages as a biocontrol tool. Phages are considered a potential biocontrol tool as they possess a number of favourable characteristics including that they are naturally occurring predators of bacteria, are harmless against mammalian cells and have widespread and easy application. However, for phage application to be successful limitations such as host resistance, lysogenic conversion and diffusion rates must be overcome through characterisation, kinetic and applied experiments. Therefore, the aim of this chapter was to investigate biocontrol potential of the *Listeria* phage CTLLm3 and the *E. coli* phage AAPEc6 through kinetic and applied experiments on biofilms and cooked roast beef samples respectively. AAPEc6 was stable in the pH range 3 to 7 and temperature range of -20 °C to 50°C, whereas CTLLm3 was stable from pH 5 to 7 and from temperatures -20 °C to 40 °C. Both phages showed optimal host lysis between 30°C and 40 °C and at MOI values of 10 and 100 *in vitro*. The application of AAPEc6 onto cooked roast beef samples resulted in a significant reduction in bacterial numbers, while CTLLm3 application significantly limited bacterial growth in biofilms. These results highlight the biocontrol potential of both phages.

3.1 Introduction

Despite the use of adequate sanitation practises and control measures including UV irradiation, pasteurisation, high pressure treatment and chemical sanitisers, foodborne pathogens continue to be detected in foods and on preparation surfaces (Sillankorva, Oliveira et al. 2012). As foods and preparation surfaces can become contaminated at various stages in the production line the risk of initial contamination and the potential for biofilm formation remains significant (Hagens and Loessner 2007). These factors combined with the continued increase in reported cases of foodborne illness have encouraged investigation into alternative methods to control initial contamination and subsequent spread (Sillankorva, Oliveira et al. 2012). One such alternative is the use of phage as a biocontrol agent (Hagens and Loessner 2007).

Phages are considered for biocontrol as they possess a number of advantages, including that they play a vital role in the control of naturally occurring bacterial populations (Hagens and Loessner 2014). They are host specific, thus they are harmless against mammalian cells (Garcia, Martinez et al. 2008). Studies in both humans and animals have not resulted in adverse effects following phage consumption, suggesting that limited consumption is harmless (Bruttin and Brüssow 2005, Hagens and Loessner 2007). Phages also have widespread applications, can be applied from the beginning to the end of food production systems, can be used as a decontamination tool for food processing surfaces and can be applied individually and in cocktails (Garcia, Martinez et al. 2008).

Despite these advantages there are limitations associated with phage use that should be considered. Such limitations include the development of bacterial resistance, diffusion rates, microbial load and lysogenic conversion following application (Hagens and Loessner 2007). To reduce the possibility of such limitations, phages considered for biocontrol should possess a number of characteristics. Firstly, phages must be replicating through the lytic lifecycle to ensure that progeny phage are quickly released therefore, surrounding bacterial populations are rapidly infected and killed (Henry and Debarbieux 2012). Secondly, phages selected for biocontrol purposes should have a relatively broad host range to minimise the possibility of developing host resistance (Strauch, Hammerl et al. 2007). Other factors to be considered prior to phage application include the efficacy of phages at the temperature that they will be used, the potential for the development of transduction and antibiotic resistance and the ability of the phage to be used in a specific food system (Hagens and Loessner 2014).

Although existing research supports the use of phages for controlling pathogen spread and phage based products such as Listex 100 and ListShield have been successfully applied in commercial environments, selected phages should be tested under conditions that foods are stored and processed at to be indicative of biocontrol potential in industrial environments (Mahony, McAuliffe et al. 2011). As external factors such as temperature, pH, salinity, surface attachment and storage influence phage functioning, the use of applied experiments and effective trials prior to commercial application provides a better understanding of phage functioning and will reduce the possibility of limitations (Sillankorva, Oliveira et al. 2012).

Thus, the aim of this series of experiments was to investigate the potential of the *Listeria* phage CTLLm3 and the *E. coli* phage AAPEc6 as biocontrol agents. Kinetic experiments included the investigation of phage lysis at varying multiplicity of infection (MOI) *in vitro* and phage stability at varying temperatures and pH. Applied experiments included the application of CTLLm3 onto *L. monocytogenes* biofilms attached onto multi-well plates and AAPEc6 onto cooked roast beef to determine biocontrol potential in industrial environments.

3.2 Materials and methods

3.2.1 Phage kinetic experiments

3.2.1.1 Phage pH stability experiment

To measure phage stability under varying pH conditions 10 ml of broth was dispensed into tubes and the pH adjusted to 3, 5, 7, and 10 using HCl and NaOH and vortexed. For the *Listeria* phage CTLLm3, TSB broth was used, whereas for the *E. coli* phage AAPEc6, LB broth was used. 0.2 ml aliquots of phage at a concentration of 10^7 pfu/ml was added to each of the broths in the pH range. Tubes were incubated at room temperature, shaking at 50 rpm for the duration of the experiment. At times 0, 1, 2, 3 and 24 hours 0.1 ml samples were taken from the tubes and diluted in 10 ml (1:100 dilution), samples were further diluted for plating. 10 µl of the diluted samples were plated using the spot plate method, and overlay method for enumeration. All plating was done in triplicate and SM buffer was used as a control. Plates were incubated at 37°C for 18 - 24 hours for *E. coli*, and 30 °C for 24 hours for *Listeria*.

3.2.1.2 Phage temperature stability experiment

The stability of the phages AAPEc6 and CTLLm3 was assessed at the temperatures -20, 4, 20, 30, 37, 40 and 50 °C. 10 ml broth was tempered to experimental temperatures before dispensing into tubes and adding 0.2 ml of phage at a concentration of 10^7 pfu/ml. Tubes were then incubated at the required temperature and 0.1 ml samples were taken at 0, 1, 2, 3 and 24 hours and samples were plated using the spot plate method. All plating was done in triplicate with SM buffer used as a control. Plates were incubated for 18 - 24 hours at 37 °C for *E. coli* and 30 °C for 24 hours for *Listeria*.

3.2.1.3 Effect of temperature on host lysis

The effect of temperature on host cell lysis was investigated using the multi-well plate reader, the multi-well plate was set up with eight replicate experiments each at an MOI of 10, one blank and three phage- free bacterial controls. For the experimental condition 0.1 ml of host and 0.1 ml phage diluted to the required concentration was added to each well, the host strain used for CTLLm3 was NZRM 3449 and the host strain used for AAPEc6 was NZRM 1345. The phage-free bacterial controls contained 0.1 ml of the host strain diluted to the required concentration and 0.1 ml of SM buffer, the blank control condition contained 0.2 ml of SM buffer. In each of the wells there was a total volume of 0.2 ml. The experiments

were run at 15, 25, 30, 37 and 40 °C, readings were taken every two minutes, for a duration of two hours and thirty minutes.

3.2.1.4 Testing various multiplicity of infection input

This experiment was undertaken using the multi-well plate reader which was set up with varying MOI ratios ranging from 0.1 to 100. The plate was set up with pre-warmed broth, for the *Listeria* phage CTLLm3 TSB was used, whereas for AAPEc6 LB was used. Each row was a replicate experiment and included one blank and three phage-free bacterial controls. The experimental condition contained 0.1 ml of host and 0.1 ml phage diluted to the required concentration, for CTLLm3 the host strain used was NZRM 3449 and for AAPEc6 the host strain used was NZRM 1345. The phage-free bacterial controls contained 0.1 ml host diluted to the required concentration and 0.1 ml SM buffer, the blank controls contained 0.2 ml SM buffer only, each well contained a total volume of 0.2 ml. Results were read every two minutes for twelve hours.

3.2.2 Applied experiments

3.2.2.1 E. coli phage AAPEc6 application onto cooked roast beef

To test the biocontrol potential of AAPEc6 the phage was tested on cooked roast beef samples at 24 °C, with each condition plated in triplicate at 0, 3, 6 and 24 hours.

Firstly, 10ml LB broth was inoculated with a colony of NZRM 1345 and grown overnight at 37°C in a shaking incubator. Cooked roast beef was aseptically cut into 2cm by 2cm squares using a sterile scalpel and three replicate samples were placed into petri dishes, before exposure to experimental temperatures. The overnight *E. coli* host culture was diluted to 10^4 CFU/ml and 20µl was pipetted onto the surface of the meat, samples were set aside for 10 minutes at room temperature to allow for host attachment.

The three control conditions set up in the experiment were host only, phage only and unadulterated meat, all were performed in triplicate per sampling time point. For phage only controls 20 µl of sterile water and 20 µl of phage was pipetted onto meat surfaces, for no addition controls 40 µl of filtered sterile water was pipetted onto the meat surface. For host only controls 20 µl of filtered sterile water was pipetted with 20 µl of the host culture.

Processing of the samples was carried out at 0, 3, 6 and 24 hours. At each time point each meat sample was aseptically transferred into a small Whirlpak bag and 5 ml of SM buffer added. Samples were placed in the stomacher machine for 2 minutes, before transferring the liquid into a 10 ml centrifuge tube and centrifuging for 10 minutes at 3000 g to pellet host cells. The supernatant was pipetted off and the pelleted bacterial cells were re-suspended in 1 ml SM buffer. 0.1 ml of the sample was removed from the tube, diluted to the appropriate concentration and plated on MacConkey agar. Meat only control samples were undiluted and plated directly onto MacConkey agar to determine the presence of any *E. coli* in the original sample.

To determine phage counts, the supernatant of phage containing samples was filtered through a 0.22 µm filter into a sterile tube. For plating, 0.1 ml of the sample was added to an overlay with 0.2 ml of the host culture, before vortexing and pouring onto pre-prepared LBA plates.

Both MacConkey and LBA plates were incubated overnight at 37 °C before plates were counted. The average bacteria count for the three replicates was divided by four, and the average phage counts divided by five to give a per cm^2 result. Significance testing was completed using Microsoft Excel and the T-TEST function, with 2-tails and assumed equal variance.

3.2.2.2 Listeria phage CTLLm3 application on biofilms

To investigate the ability of the *Listeria* phage CTLLm3 to limit the growth of *L. monocytogenes* in biofilms, the phage was applied with two different bacterial strains to 96 multi-well plates before incubating and staining using the crystal violet assay to determine bacterial concentrations. The *L. monocytogenes* strains used in this experiment were NZRM 3449 and NZRM 3370 as previous host range testing suggested that CTLLm3 was lytic on both strains, producing small clear plaques when tested on bacterial lawns.

Firstly, two lots of 5 ml TSB broth was inoculated with a single colony of NZRM 3449 and NZRM 3370 before incubating overnight at 24 °C. Following this, 150 µl of HTM minimal media was loaded into each of the wells, before adding 15 µl of the overnight bacterial culture and 15 µl of the phage at a concentration of 10^7 PFU/ml. Each experimental condition was carried out in quadruplicate, with each plate containing at least 16 wells loaded with 150 µl of HTM buffer as un-infected controls and 16 wells loaded with 150 µl HTM and 15µl of the bacterial culture as bacteria only controls.

The plate was then covered with the lid and sealed with parafilm to prevent evaporation before incubating at 30 °C for 72, 96, 120, 144 and 168 hours. After incubation cultures were removed and wells washed twice with PBS buffer by gently pipetting up and down. 170 µl of aqueous crystal violet solution was added to each well, before incubating for 15 minutes at room temperature. The crystal violet solution was then removed and the wells washed twice with PBS buffer by gently pipetting up and down, the plate was then air dried. To dissolve the biofilm 170 µl of 95% ethanol was added to each of the wells and left for one hour. 125 µl from each well was then transferred into a new multi-well plate and the concentration of crystal violet was determined by measuring OD readings at 600nm. To correct for background staining, mean OD values from uninoculated controls were subtracted by mean OD values from the experimental condition, before a two-tailed t-test for significance was calculated using Microsoft Excel T-TEST function with 2-tails and assumed equal variance.

3.3 Results

3.3.1 Stability and kinetic experiments

3.3.1.1 pH stability

An important consideration for biocontrol in foods and food processing environments is pH stability, as phages may be exposed to pH environments that are different to those where they have been isolated. To determine the stability of the phages at different pH levels, phages were inoculated into broths adjusted to different pHs and survival measured up to 24 hours. AAPEc6 retained lytic potential at pH 3, 5, and 7 for 2 hours, however no plaques were recovered at pH 10 at any sampling point (0-24 hours) (Table 3.1). After 3 hours the phage remained stable at pH 3 and 5, although the plaques observed from these assays were cloudy. AAPEc6 was very stable at neutral pH with clear plaques recovered at all time-points (Table 3.1).

Table 3.1: AAPEc6 stability at pH 3, 5, 7 and 10 at times 0, 1, 2, 3 and 24 hours

Sampling Time (Hours)	pH 3	pH5	pH7	pH10
0	(+)	(+)	(+)	(-)
1	(+)	(+)	(+)	(-)
2	(+)	(+)	(+)	(-)
3	(+)	(-)	(+)	(-)
24	(0)	(0)	(+)	(-)

(+) = Lysis using spotting technique, (-) = No plaques, (0) = Opaque/cloudy plaques

The data obtained for CTLLm3 were different to those observed for AAPEc6. CTLLm3 was very stable at pH 5 and 7, surviving up to 24 hours incubation. However, no plaques were observed at pH 3 or 10 at any of the time points (Table 3.2). No cloudy plaques were observed in these assays.

Table 3.2: CTLLm3 stability at pH 3, 5, 7 and 10 at times 0, 1, 2, 3 and 24 hours

Sampling Time (Hours)	pH 3	pH5	pH7	pH10
0	(-)	(+)	(+)	(-)
1	(-)	(+)	(+)	(-)
2	(-)	(+)	(+)	(-)
3	(-)	(+)	(+)	(-)
24	(-)	(+)	(+)	(-)

(+) = Lysis using spotting technique, (-) = No plaques, (0) = Opaque/cloudy plaques

3.3.1.2 Temperature stability

The ability of phages to remain stable across a range of temperatures is crucial for the purpose of biocontrol, as phages may be exposed to varying temperatures within the applied environment. Therefore, the stability and lytic potential of AAPEc6 and CTLLm3 was investigated through the incubation of phages at a range of temperatures in broth. Broths were inoculated with the phages and tempered to the experimental conditions before sampling at 0, 1, 2, 3 and 24 hours. AAPEc6 was stable at all temperatures tested (-20 to 50 °C) for up to 24 hours (Table 3.3).

Table 3.3: AAPEc6 stability at temperatures -20, 4, 25, 30, 37, 40 and 50 °C at times 0, 1, 2, 3 and 24 hours

Sampling Times (Hours)	-20 °C	4 °C	25 °C	30 °C	37 °C	40 °C	50 °C
0	(+)	(+)	(+)	(+)	(+)	(+)	(+)
1	(+)	(+)	(+)	(+)	(+)	(+)	(+)
2	(+)	(+)	(+)	(+)	(+)	(+)	(+)
3	(+)	(+)	(+)	(+)	(+)	(+)	(+)
24	(+)	(+)	(+)	(+)	(+)	(+)	(+)

(+) = Lysis using spotting technique, (-) = No plaques, (0) = Opaque/cloudy plaques

The temperature stability data obtained for CTLLm3 was similar to the results gathered for AAPEc6 and suggested that CTLLm3 retained lytic potential from -20 to 37 °C for up to 24 hours. However, unlike AAPEc6, CTLLm3 was stable at 40 °C for up to 3 hours and did not show lytic potential at 24 hours. When CTLLm3 was incubated at 50 °C no plaques were observed at any of the sampling times (Table 3.4).

Table 3.4: CTLLm3 stability at temperatures -20, 4, 25, 30, 37, 40 and 50 °C at times 0, 1, 2, 3 and 24 hours

Sampling Times (Hours)	-20 °C	4 °C	25 °C	30 °C	37 °C	40 °C	50 °C
0	(+)	(+)	(+)	(+)	(+)	(+)	(-)
1	(+)	(+)	(+)	(+)	(+)	(+)	(-)
2	(+)	(+)	(+)	(+)	(+)	(+)	(-)
3	(+)	(+)	(+)	(+)	(+)	(+)	(-)
24	(+)	(+)	(+)	(+)	(+)	(-)	(-)

(+) = Lysis using spotting technique, (-) = No plaques, (0) = Opaque plaques

3.3.1.3 Host cell lysis at varying temperatures

The changing nature of industrial food processing environments and the use of temperature-controls on foods during processing means that the ability of phages to lyse host cells at varying temperatures is crucial for the purpose of biocontrol. To investigate the potential of the phages AAPEc6 and CTLLm3 to lyse host cells under a range of temperatures the phages were incubated with their hosts in broth at temperatures from 25 to 50°C.

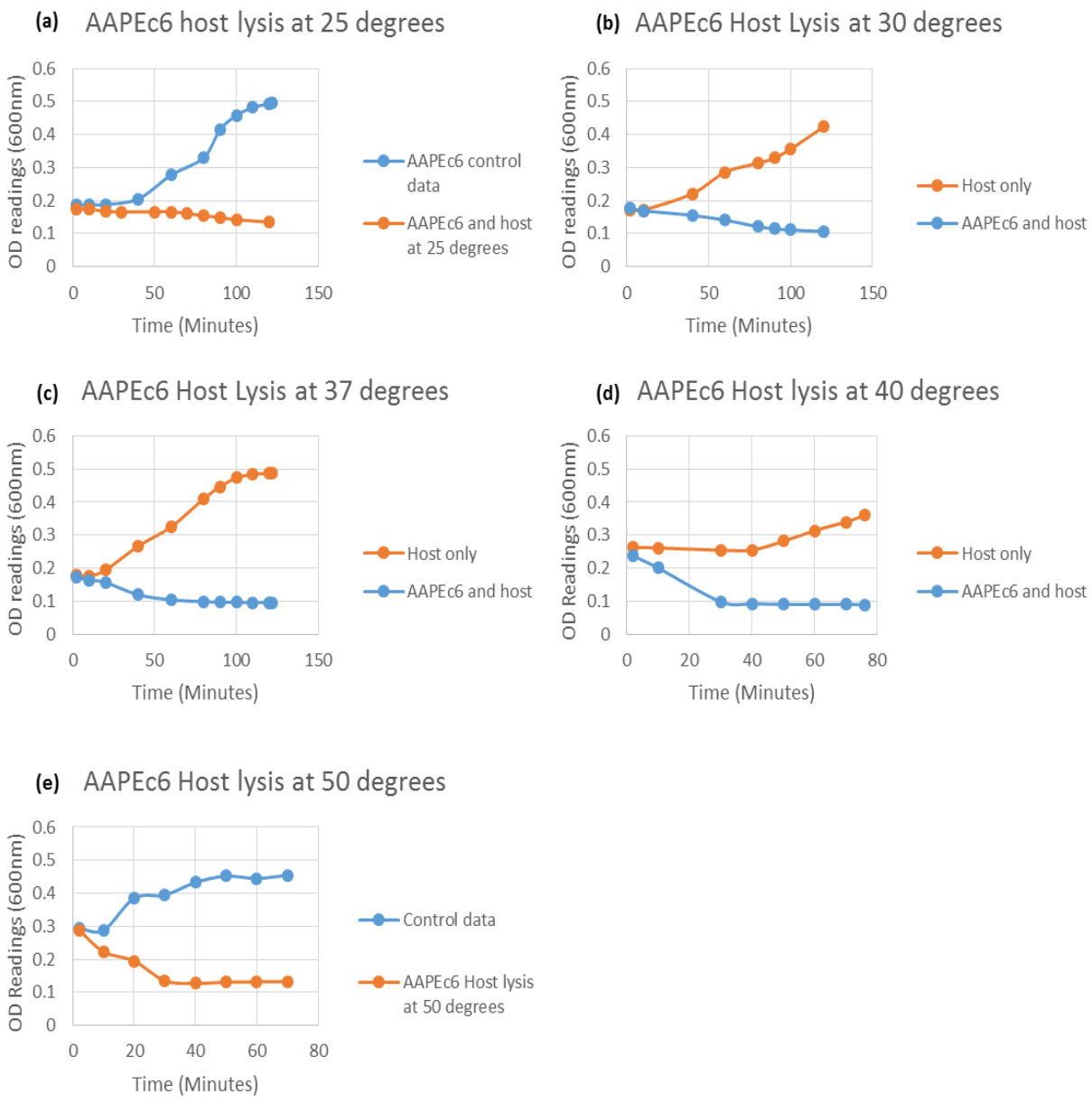

Figure 3.1: Lysis of *E. coli* host NZRM 1345 by *E. coli* phage AAPEc6 over a period of 120 minutes at temperatures of (a) 25, (b) 30, (c) 37, (d) 40 and (e) 50 °C

Host lysis of NZRM 1345 by AAPEc6 was observed at 25, 30, 37, 40 and 50 °C (Figure 3.1). However, optimal host lysis occurred at 37 and 40 °C. At these temperatures a rapid decrease in OD readings was observed throughout the experiment, suggesting efficient host lysis. At temperatures of 25, 30 and 50 °C a lag period was observed during the initial phase of the experiment suggesting minimal host lysis, following this OD readings began to steadily decrease indicating continual host lysis over the remaining duration of the experiment (Figure 3.1).

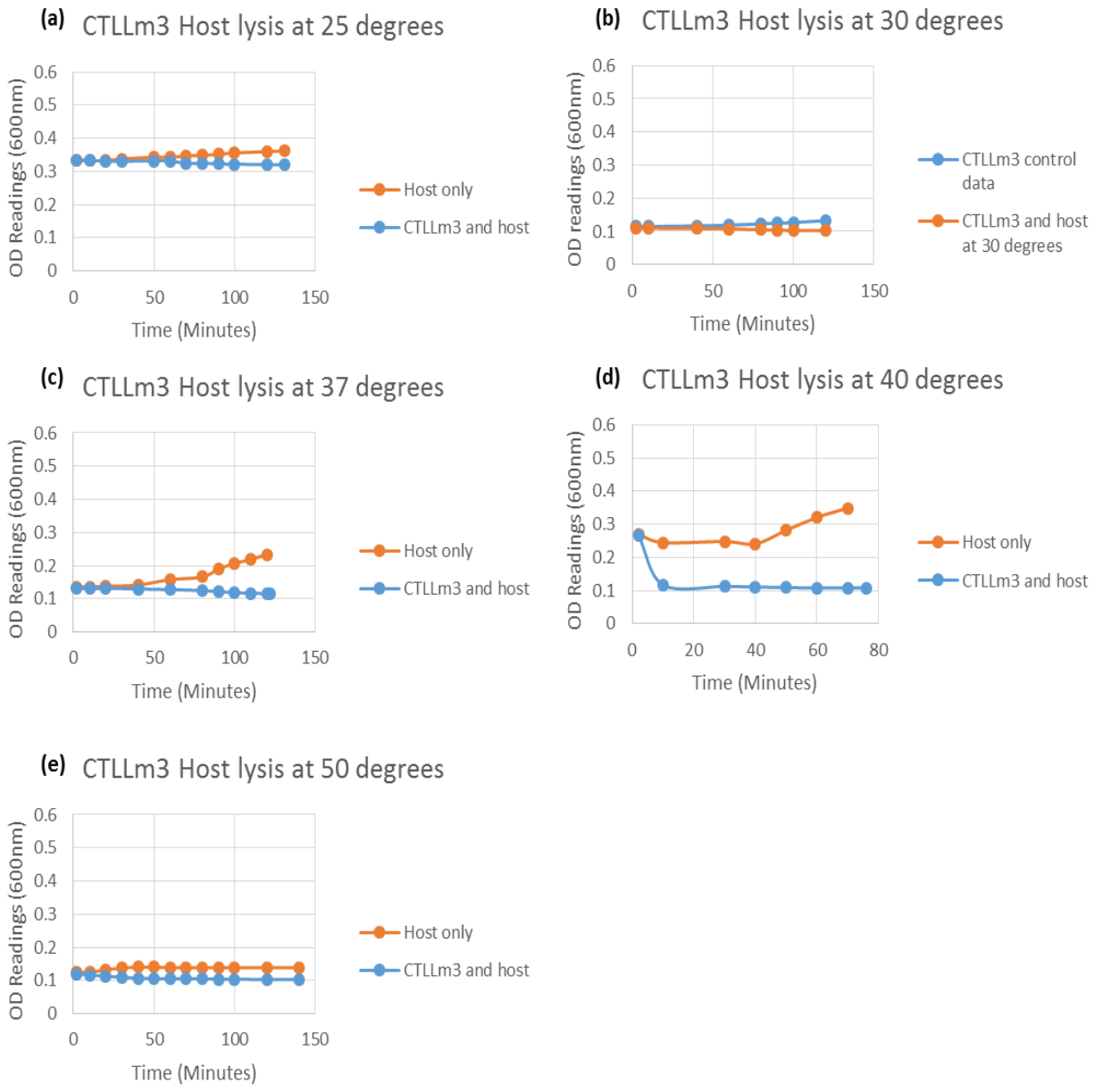

Figure 3.2: Lysis of *L. monocytogenes* host NZRM 3449 by *Listeria* phage CTLLm3 over a period of 120 minutes at temperatures of (a) 25, (b) 30, (c) 37, (d) 40 and (e) 50 °C.

Likewise, when lysis of NZRM 3449 by CTLLm3 was investigated results suggested that host cell lysis occurred at temperatures of 25, 30, 37, 40 and 50 °C. Optimal lysis of NZRM 3449 was observed at 30, 37 and 40 °C, at these temperatures OD readings rapidly decreased indicating efficient host lysis. At temperatures of 25 and 50 °C limited lysis was observed during the initial stage of the experiment before OD readings began to gradually decrease suggesting the occurrence of steady, continual host lysis (Figure 3.2).

3.3.1.4 Host cell lysis at varying MOI in vitro

To optimise phage application in industrial environments an understanding of phage and bacterium interactions should be considered. A critical aspect to this is the ration of phages to host cells (MOI) required for successful infection. To investigate this, host cells were infected in broth at varying MOI *in vitro* and growth of the host cells was monitored by optical density.

The readings obtained over the course of the experiment suggested that the optimal MOI for AAPEc6 was at 10 and 100 (Figure 3.3). At the higher MOI's tested the density of the host declined rapidly after the experiment started, indicating efficient lysis. Lower MOI's resulted in initial retardation of growth followed by a decrease in cell density compared to the controls. However, all MOI's tested resulted in approximately the same reduction in cell density in the phage treatment by the end of the experiment.

Similarly, the readings obtained for CTLLm3 suggest that optimal host lysis occurred at an MOI of 10 and 100. At these concentrations a rapid decrease in OD readings was observed, indicating efficient lysis of the host. Whereas, at MOI concentrations of 0.1 and 1 limited host lysis was observed. At these lower MOI's OD readings steadily increased over the duration of the experiment, however, this increase did not exceed the increase observed in the in the host only control condition, suggesting a limited degree of host lysis at these concentrations (Figure 3.4).

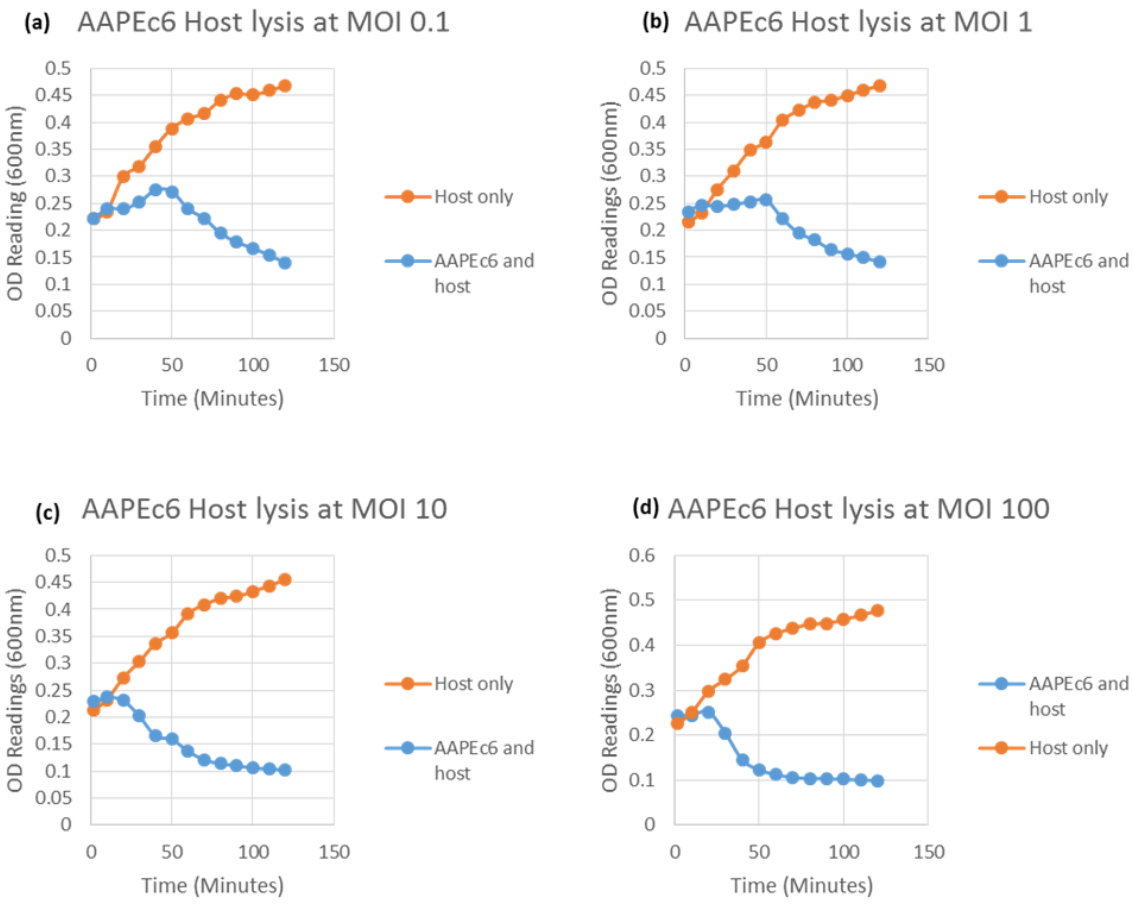

Figure 3.3: Host lysis of *E. coli* NZRM 1345 by AAPEc6 at varying MOI (multiplicity of infection) *in vitro* at 37 °C over a period of 120 minutes. (a) AAPEc6 host lysis at MOI of 0.1 *in vitro*, (b) AAPEc6 host lysis at MOI of 1 *in vitro*, (c) AAPEc6 host lysis at MOI of 10 *in vitro*, (d) AAPEc6 host lysis at MOI of 100 *in vitro*.

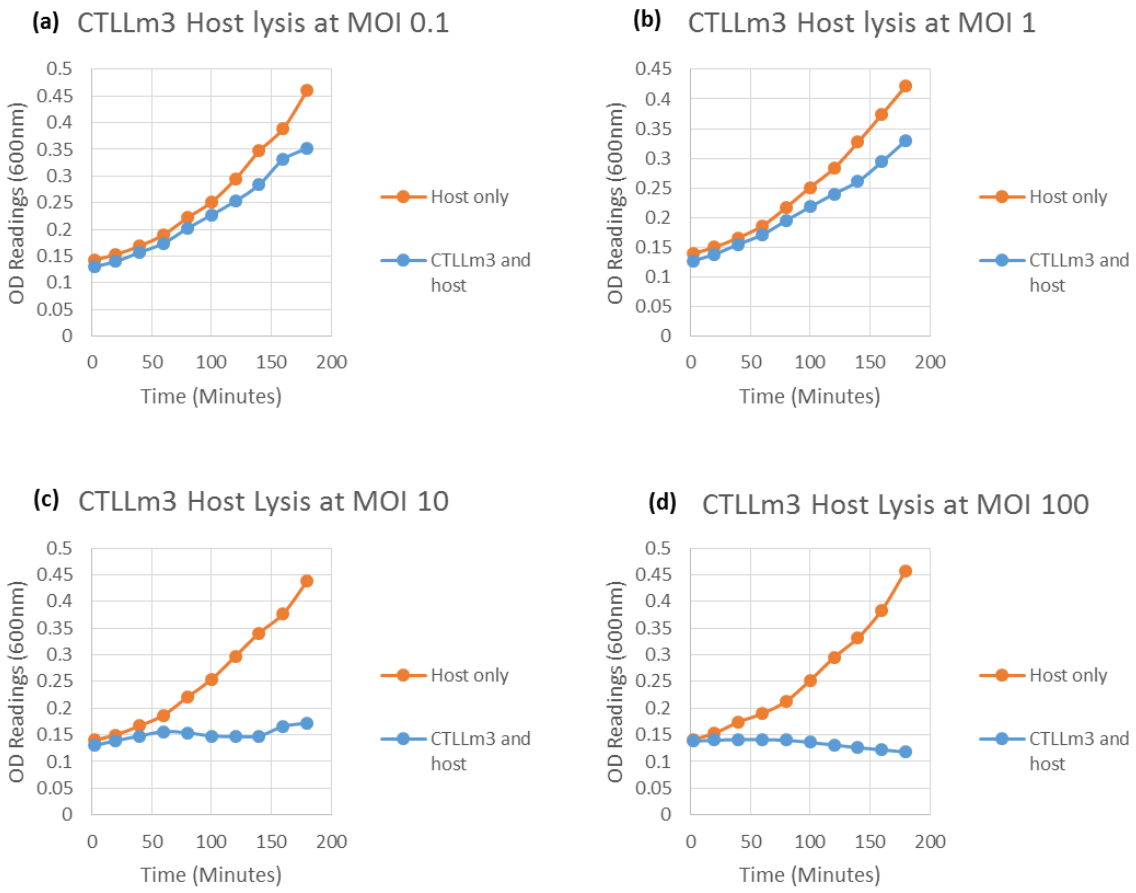

Figure 3.4: Host lysis of *L. monocytogenes* NZRM 3449 by CTLLm3 at varying multiplicity of infection (MOI) *in vitro* at 37 °C over a period of 120 minutes. (a) CTLLm3 host lysis at MOI 0.1 *in vitro*, (b) CTLLm3 host lysis at MOI 1 *in vitro*, (c) CTLLm3 host lysis at MOI 10 *in vitro*, (d) CTLLm3 host lysis at MOI 100 *in vitro*.

3.3.2 Applied Experiments

3.3.2.1 E. coli phage AAPEc6 application on meats

As environmental factors can significantly influence phage efficacy, applied experiments must be conducted in controlled conditions which are similar to the intended industrial use prior to deployment in industrial environments. Therefore, the biocontrol potential of AAPEc6 was investigated by seeding cooked roast beef samples with *E. coli* NZRM 1345 and measuring the effect of applied phages on viability of the pathogen for up to 24 hours.

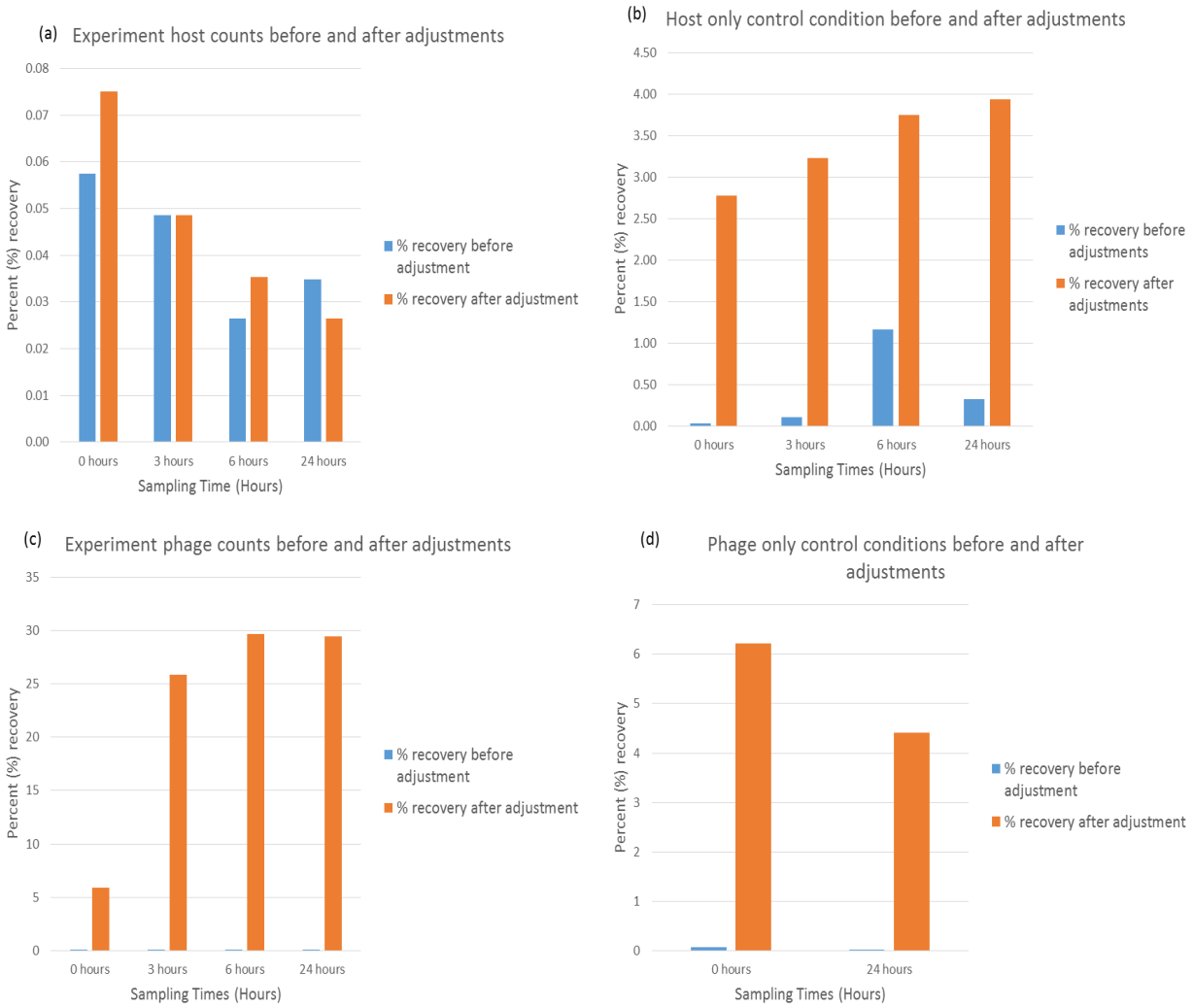

Figure 3.5: Comparison of percent (%) recovery of AAPEc6 and *E. coli* NZRM 1345 from MacConkey agar before and after protocol adjustments. (a) Host only control, (b) Experiment host counts, (c) Phage only control, (d) Experiment phage counts.

The results of the initial experiments conducted suggested recovery of both phage and bacteria was suboptimal, therefore adjustments to the protocol were made including the use of BHI instead of LB, increasing stomaching time and altering phage and bacteria concentrations. Following these adjustments an improvement in recovery rate for both phage and bacteria was observed (Figure 3.5).

A two-tailed t-test analysis suggested that the application of AAPEc6 at a concentration of 10^8 PFU/piece achieved a significant reduction in numbers of *E. coli* NZRM 1345 from 0 to 24 hours ($p < 0.05$). Following the application of AAPEc6 onto contaminated meat samples a reduction of up to 2.17 $\log_{10}$ in the experimental treatment compared to the control was observed (Table 3.5; Figure 3.6). With the difference in *E. coli* NZRM 1345 counts between control and phage treated roast beef samples increasing with time. Meanwhile, AAPEc6 numbers significantly increased from 0 to 24 hours ($p < 0.05$).

Table 3.5: Effect of AAPEc6 application onto roast beef samples contaminated with *E. coli* NZRM 1345 from 0 to 24 hours

Sampling Time (Hours)	Log difference (cm^2)
0 hours	1.57
3 hours	1.82
6 hours	2.03
24 hours	2.17

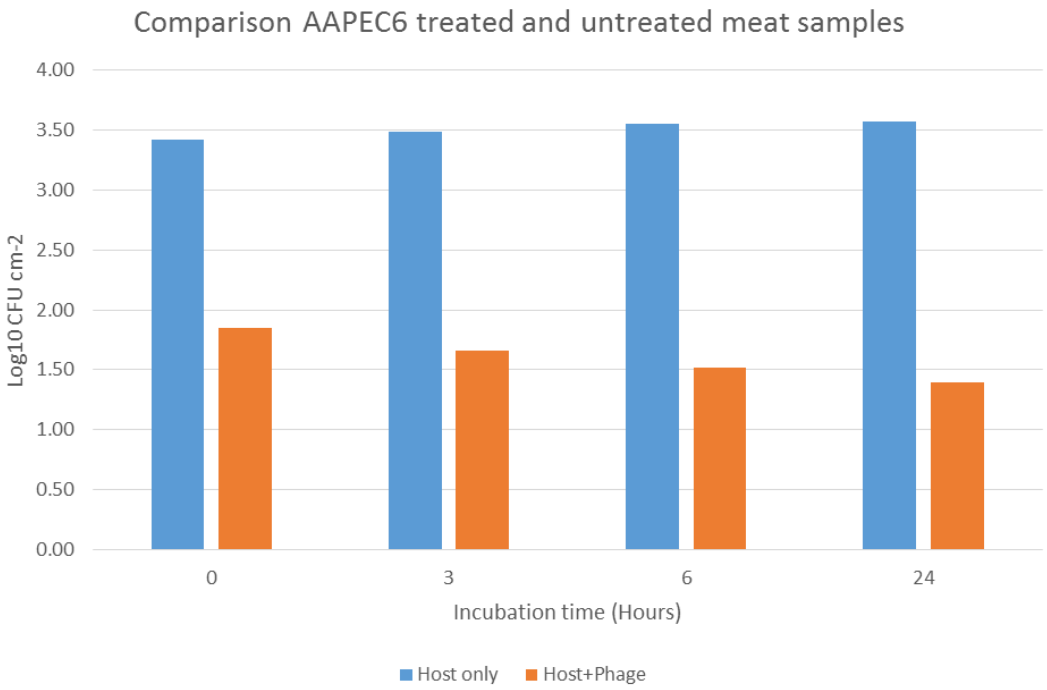

Figure 3.6: Comparison of AAPEc6 activity in treated and untreated samples over a period of 0, 3, 6 and 24 hours at 37 °C.

3.3.2.2 Listeria phage CTLLm3 application on biofilms

The potential for CTLLm3 to limit the development of biofilms in industrial environments was investigated by applying the phage with the two *L. monocytogenes* strains NZRM 3449 and NZRM 3370 to multi well plates before incubating for 72, 96, 120, 144 and 168 hours at 30 °C and staining using the crystal violet assay to determine OD readings for each well.

The results of the two-tailed t-test suggested that at 72, 96 and 120 hours CTLLm3 was able to significantly reduce the growth of both *L. monocytogenes* strains NZRM 3449 and NZRM 3370 ($p < 0.05$). However, at 144 and 168 hours CTLLm3 application did not result in a significant reduction in biofilm mass ($p > 0.05$) as shown in Table 3.6; Figure 3.7 and 3.8.

Table 3.6: CTLLm3 activity on biofilms composed of *L. monocytogenes* strains NZRM 3449 and 3370 and grown for 72, 96, 120, 144 and 168 hours at 30 °C.

Biofilm growth time (Hours)	**OD readings difference values between control and experimental conditions**			
	3370 host only	**3370 host and CTLLm3**	**3449 host only**	**3449 host and CTLLm3**
72	0.362	0.147	0.105	0.154
96	0.256	0.051	0.120	0.200
120	0.107	0.147	0.129	0.153
144	0.096	0.079	0.131	0.133
168	0.103	0.088	0.124	0.157

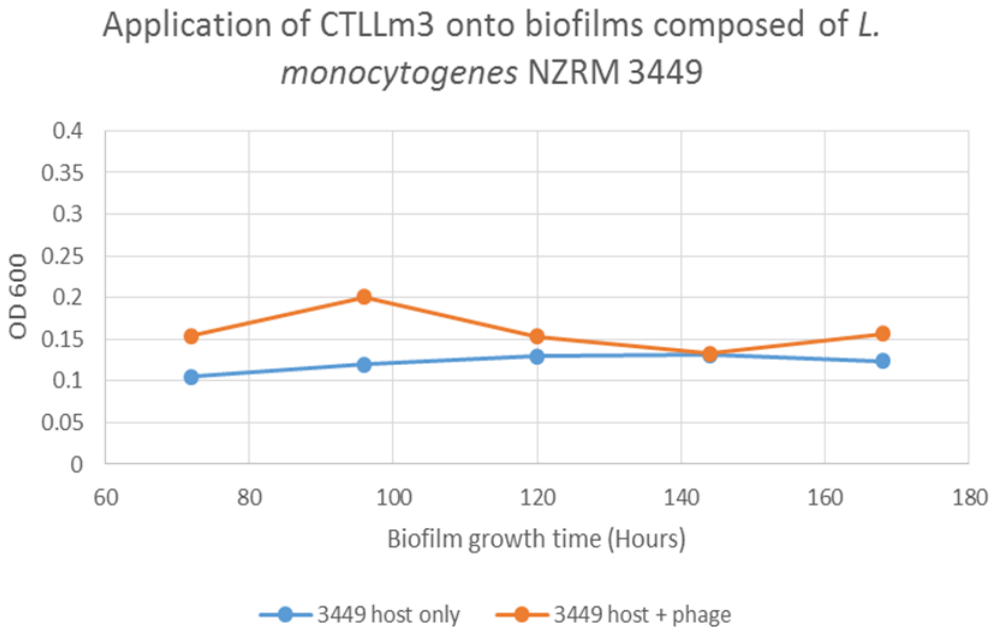

Figure 3.7: CTLLm3 activity following application with *L. monocytogenes* strain NZRM 3449 onto biofilms incubated for 72, 96, 120, 144 and 168 hours at 30 °C.

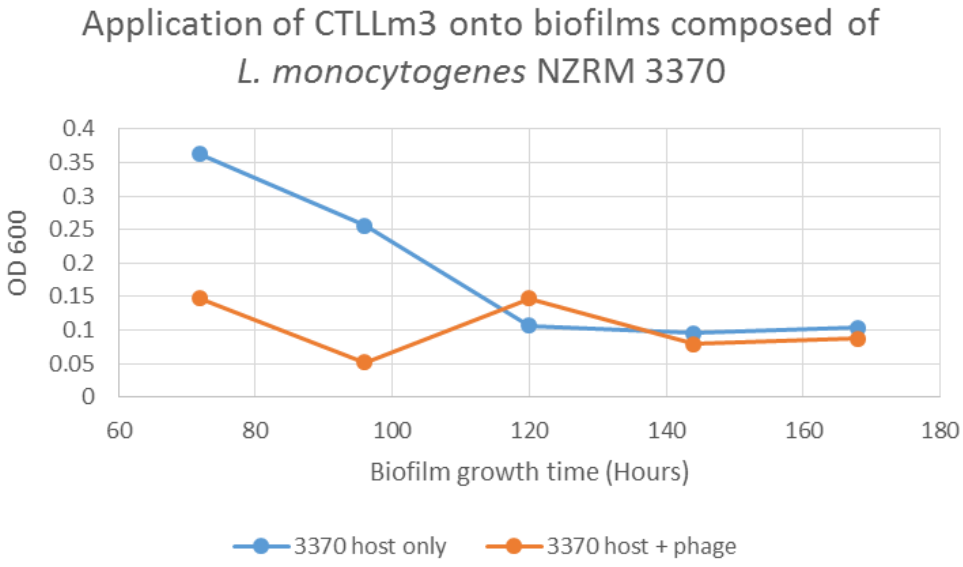

Figure 3.8: CTLLm3 activity following application with *L. monocytogenes* strain NZRM 3370 onto biofilms incubated for 72, 96, 120, 144 and 168 hours at 30 °C.

3.4 Discussion

3.4.1 pH stability

The results of the pH stability experiment suggest that AAPEc6 did not show lytic potential at pH 10, indicating instability under alkali conditions. However, lysis was observed between pH 3 and 7 suggesting stability in acidic and neutral conditions from 0 to 24 hours. Although AAPEc6 showed lytic potential at 24 hours under acidic conditions, the plaques produced were opaque and cloudy, this change in plaque morphology could be the result of a stress response to unfavourable conditions (Seaman and Day 2007). These results are in agreement with previous studies which suggest that *E. coli* phage have an optimum pH of 6 and generally show decreasing viability as conditions become more alkali (Jończyk, Kłak et al. 2011). The high stability of AAPEc6 across a range of pH, suggests that the phage has the potential to be successfully applied in foods and preparation surfaces that are exposed to acidic and neutral environments, as the phage remains stable and lytic under these conditions.

Similarly, the *Listeria* phage CTLLm3 showed lytic ability between pH 5 and 7 using the spot plate method, however, when tested at pH 3 and 10 no plaques were observed, suggesting instability in highly acidic and alkali conditions. The stability of the phage between a pH of 5 and 7 is advantageous for the purpose of biocontrol as *L. monocytogenes* can grow at pH levels between 5 and 9 (Herald and Zottola 1988). The ability of CTLLm3 to remain stable and active in mildly acidic to neutral conditions suggests that the phage has the potential to be applied on surfaces and cleaning products within the pH range 5-7.

3.4.2 Temperature stability

Temperature influences the processes of attachment, penetration and length of latent period, therefore plays a vital role in phage survival (Jończyk, Kłak et al. 2011). At higher temperatures the latent period is extended, whereas, at lower temperatures a decreased concentration of phage genetic material penetrates into the host cell resulting in reduced phage multiplication (Tey, Ooi et al. 2009). Previous studies investigating phage stability at various temperatures have suggested that the ability of phages to remain stable in unfavourable temperatures is diverse within and among phage families (Jończyk, Kłak et al. 2011).

The results of the temperature stability experiment indicated that AAPEc6 retains lytic potential at temperatures from -20 to 50 °C. Similarly, the *Listeria* phage CTLLm3 was stable

from -20 to 40 °C however, lytic potential was not observed at temperatures exceeding 40 °C indicating instability or inactivation of the virion at higher temperatures. The ability of CTLLm3 and AAPEc6 to remain stable and viable across a broad range of temperatures is useful for the purpose of biocontrol as it allows for potential application across a variety of industrial environments, including freezers, room temperature conditions and warmer storage conditions.

3.4.3 Host lysis at varying temperatures

The results of the experiment for both AAPEc6 and CTLLm3 suggested that the optimum temperature for host cell lysis was between 30 and 40 °C which is within the optimum growth temperature for both *L. monocytogenes* and *E. coli*. Between 30 and 40 °C, OD readings rapidly decreased to a level similar to blank readings, whereas phage-free bacteria controls continued to steadily increase as the experiment continued. As the temperature increases to optimum host conditions, host cell density increases as bacterial cells rapidly multiply. This rise in host cell density is likely to increase phage activity, thus infection (Shao and Wang 2008).

When the experiment was carried out at 50 °C OD readings for the experimental condition decreased before stabilising, suggesting lysis of both *L. monocytogenes* and *E. coli*. However, OD readings for the control condition increased between 0.15-0.2 before stabilising, suggesting the growth of *E. coli* and *Listeria* at 50 °C, although neither bacterium is known to grow at temperatures exceeding 46 °C (Roberts and Wiedmann 2003, Farrokh, Jordan et al. 2013). This observed increase may be attributed to residual growth of the inoculum or inaccuracies in the measurement of the temperature within the culture well.

3.4.4 Host lysis at varying MOI

There are a number of factors that should be considered prior to phage application including surface structure, phage diffusion ability, amount of fluid present and the target reduction levels required however, among the most important is the absolute concentrations of phage and bacteria to be applied (Hagens and Loessner 2010). Cellular multiplicity of infection, or MOI is defined as the mean number of virions successfully infecting a population of bacterial cells (Zwart, Tromas et al. 2013). MOI is a crucial parameter for describing interactions between virions and bacterial cells, predicting the dynamics of mixed-genotype interactions and for considering viral evolution (Zwart, Tromas et al. 2013).

The results of the experiment for AAPEc6 suggested that greatest host lysis occurred at MOI values of 10 and 100 *in vitro* with OD readings decreasing to levels similar to blanks over a shorter period of time when compared to bacteria only controls. At MOI values of 0.1 and 1 AAPEc6 showed significant host lysis as OD readings continued to decrease throughout the experiment.

Like AAPEc6, CTLLm3 showed optimal host lysis at an MOI of 10 and 100 *in vitro* as OD readings decreased to levels similar to blanks over a shorter period of time when compared to bacteria only controls which continued to show increased OD readings throughout the duration of the experiment. However, at MOI values of 0.1 and 1 OD readings for the experimental condition generally increased over the duration of the experiment, however did not exceed the bacteria only control condition, suggesting limited host lysis.

These results suggest that when applied at the appropriate MOI both phages have the potential to reduce host concentrations. If phages are to be applied to liquid foods low initial concentrations are required as phages have the potential to achieve sufficient host lysis in a short period of time, due to an increased likelihood of bacteria and phage interactions within the liquid medium (Hagens & Loessner, 2010). However, if phages are to be applied to solid foods it is likely that a higher phage concentration will be required to increase the chance of phage and bacteria interactions, thus host lysis (Hagens & Loessner, 2010). For optimal phage activity to be achieved following application the specific environment and food type to which the phages are to be applied should be further considered.

3.4.5 E. coli Phage AAPEc6 application on meat

Initial experiments suggested limited recovery of both bacteria and phage, to overcome this several adjustments to the protocol were made, including the use of BHI instead of LB broth, increasing stomaching time and altering phage and bacteria concentrations, with the recovery rate improving following these changes. The application of AAPEc6 onto cooked roast beef samples at a concentration of 10^8 PFU/ml resulted in up to 2.17 $\log_{10}$ reduction in concentrations of *E. coli* NZRM 1345 from 0 to 24 hours. From 0 to 24 hours AAPEc6 numbers increased, suggesting that over the 24 hour period phages are replicating as they come into contact with host cells within the meat matrix. These results highlight the biocontrol potential of the phage.

Previous studies investigating the use of phages to reduce bacterial numbers in applied experiments have shown similar results. A study by Liu *et al* (2015) investigated the potential

of four phages individually and in a cocktail to reduce levels of *E. coli* O157 on beef samples at temperatures of 4, 22 and 37 °C. The results of their study suggested that pathogen inactivation was greatest at higher incubation temperatures, MOI and phage exposure and that the application of phages both individually and in a cocktail resulted in a decrease in pathogen numbers. However, complete pathogen removal was not observed. A further study by Hudson *et al* (2013) isolated, characterised and applied an *E. coli* phage capable of killing the strain O157:H7. The results of the study suggested that the application of the phage in broth, on thinly sliced meats and under hot boning and conventional carcass cooling resulted in pathogen inactivation. These results suggest that there is potential for phage application in industrial environments, however, more information regarding phage sequences and phage cocktails is required if optimal application is to be achieved. The increasing availability of sequencing technology is beneficial for phage biocontrol as it can provide information regarding the suitability of phages for biocontrol application, for example, identifying the presence of toxigenic or harmful genes and determining phage stability (Mahony, McAuliffe et al. 2011). Such information is vital as it is likely to reduce the possibility of adverse effects following industrial application (Mahony, McAuliffe et al. 2011). Similarly more information regarding phage cocktails will further contribute to existing information concerning the likelihood of resistance following application and the interaction between bacteria and phages within the intended industry (Greer 2005).

3.4.6 Listeria phage CTLLm3 application on Listeria biofilms

At 72, 96 and 120 hours CTLLm3 was able to significantly limit the growth of the *L. monocytogenes* strains NZRM 3449 and NZRM 3370, with the greatest reduction in biofilm mass occurring at 72 and 96 hours. However, at 144 and 168 hours no significant reduction in biofilm mass was observed for either strain suggesting that optimal growth of the bacterium had been exceeded, therefore phage activity was limited. These results suggest that CTLLm3 has the potential to be applied in industrial environments, and would be most effective during the early stages of biofilm formation.

In a study by Soni *et al (*2010), the *Listeria* phage P100 was applied to several strains *of L. monocytogenes* on biofilms. The results from their study suggested that phage application resulted in a significant reduction in the concentrations of *Listeria* present under biofilm conditions, despite serotype, growth condition and level of biofilm development. However, for phage application to be effective in industrial environments more research into the

effectiveness of phages in mixed species biofilms and phage cocktails should be completed (Soni and Nannapaneni 2010).

3.5 Conclusion

The results of the kinetic and applied experiments suggested that CTLLm3 and AAPEc6 were stable across a range of pH and temperatures. The application of CTLLm3 onto biofilms resulted in a significant reduction in biofilm mass, meanwhile the application of AAPEc6 on cooked meat samples seeded with *E. coli* resulted in a significant reduction in bacterial numbers. These results highlight the biocontrol potential of both phages and suggest that APPEc6 and CTLLm3 have the potential to be applied in meat based environments and biofilm environments respectively.

3.6 References:

Bruttin, A. and H. Brüssow (2005). "Human volunteers receiving Escherichia coli phage T4 orally: a safety test of phage therapy." Antimicrobial agents and chemotherapy **49**(7): 2874-2878.

Farrokh, C., et al. (2013). "Review of Shiga-toxin-producing Escherichia coli (STEC) and their significance in dairy production." International journal of food microbiology **162**(2): 190-212.

Garcia, P., et al. (2008). "Bacteriophages and their application in food safety." Letters in applied microbiology **47**(6): 479-485.

Greer, G. G. (2005). "Bacteriophage control of foodborne bacteria." Journal of Food Protection® **68**(5): 1102-1111.

Hagens, S. and M. J. Loessner (2007). "Application of bacteriophages for detection and control of foodborne pathogens." Applied Microbiology and Biotechnology **76**(3): 513-519.

Hagens, S. and M. J. Loessner (2014). "Phages of Listeria offer novel tools for diagnostics and biocontrol." Frontiers in microbiology **5**.

Henry, M. and L. Debarbieux (2012). "Tools from viruses: bacteriophage successes and beyond." Virology **434**(2): 151-161.

Herald, P. J. and E. A. Zottola (1988). "Attachment of Listeria monocytogenes to stainless steel surfaces at various temperatures and pH values." Journal of Food science **53**(5): 1549-1562.

Jończyk, E., et al. (2011). "The influence of external factors on bacteriophages—review." Folia microbiologica **56**(3): 191-200.

Mahony, J., et al. (2011). "Bacteriophages as biocontrol agents of food pathogens." Current Opinion in Biotechnology **22**(2): 157-163.

Roberts, A. and M. Wiedmann (2003). "Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis." Cellular and Molecular Life Sciences CMLS **60**(5): 904-918.

Seaman, P. F. and M. J. Day (2007). "Isolation and characterization of a bacteriophage with an unusually large genome from the Great Salt Plains National Wildlife Refuge, Oklahoma, USA." FEMS microbiology ecology **60**(1): 1-13.

Shao, Y. and N. Wang (2008). "Bacteriophage adsorption rate and optimal lysis time." Genetics **180**(1): 471-482.

Sillankorva, S. M., et al. (2012). "Bacteriophages and their role in food safety." International journal of microbiology **2012**.

Soni, K. A. and R. Nannapaneni (2010). "Removal of Listeria monocytogenes biofilms with bacteriophage P100." Journal of Food Protection® **73**(8): 1519-1524.

Strauch, E., et al. (2007). "Bacteriophages: new tools for safer food?" Journal für Verbraucherschutz und Lebensmittelsicherheit **2**(2): 138-143.

Tey, B. T., et al. (2009). "Production of fusion m13 phage bearing the disulphide constrained peptide sequence (C-WSFFSNI-C) that interacts with hepatitis B core antigen." J African Biotechnol **8**: 268-273.

Zwart, M. P., et al. (2013). "Model-selection-based approach for calculating cellular multiplicity of infection during virus colonization of multi-cellular hosts."

Chapter 4: Conclusion

The results of this study further contribute to existing research regarding the application of phages as biocontrol agents against foodborne pathogens. In this study a total of five phages with biocontrol potential were assessed, three *E. coli* phages and two *Listeria* phages. Of the three *E. coli* phages, two were newly isolated from faecal matter and were named CJNEc1 and CJNEc2, the third, AAPEc6 was sourced from the laboratory collection along with the two *Listeria* phages CTLLm3 and A511.

Biocontrol potential of the phages was assessed through characterisation experiments, with results suggesting that all three *E. coli* phages had a narrow host range and were fast adsorbing. Based on electron microscopy, the two newly isolated *E. coli* phages CJNEc1 and CJNEc2 are putatively members of the family *Myoviridae,* whereas the third phage, AAPEc6 is a putative member of the family *Podoviridae.* The two *Listeria* phages had a broad host range and were fast adsorbing, based on electron microscopy images CTLLm3 was designated as a member of the *Siphoviridae* family. As the results of the characterisation studies suggested that the phages AAPEc6 and CTLLm3 showed biocontrol potential both phages were prepared for genome sequencing. However, due to time constraints the results were not obtained in time to be included in this thesis.

The biocontrol potential of AAPEc6 and CTLLm3 was further investigated through kinetic and applied experiments. AAPEc6 was stable between a pH range of 3 to 7, and a temperature range of -20 to 50°C. The phage showed optimal host lysis between 30 and 40°C and at MOI values of 10 and 100 *in vitro*. Following AAPEc6 application onto cooked roast beef samples a 2.17 $\log_{10}$ reduction in numbers of the host strain NZRM 1345 was observed from 0 to 24 hours. These results suggest that AAPEc6 has the potential to be applied in industrial, meat based environments. However, to optimise application and avoid limitations such as the development of host resistance, this phage should be applied as part of a cocktail.

CTLLm3 was stable between a pH range of 5 to 7 and a temperature range of -20 to 40°C. Like AAPEc6, CTLLm3 showed optimal host lysis between 30 and 40°C and at MOI values of 10 and 100 *in vitro*. The application of CTLLm3 with strains of *L. monocytogenes* resulted in a reduction in biofilm mass at 72, 96 and 120 hours, with the greatest reduction occurring at 72 and 96 hours. These results suggest that CTLLm3 has the potential to be applied in

industrial environments susceptible to biofilm formation, especially during the initial stages of biofilm development.

The continued detection of pathogens on foods and preparation surfaces combined with the increase in the number of reported cases of foodborne illness continues to pose a significant health threat (Hagens and Loessner 2007). To meet the challenge of reducing the global burden of foodborne disease, new technologies are required. In this work, the potential for the phages AAPEc6 and CTLLm3 to be applied in the food industry is demonstrated. However, more work regarding the interactions between phage and bacteria and the application of phages individually, and in cocktails, would need to be completed to optimise application in commercial environments.

4.1 References:

Hagens, S. and M. J. Loessner (2007). "Application of bacteriophages for detection and control of foodborne pathogens." Applied Microbiology and Biotechnology **76**(3): 513-519.

A1. Appendix:

General Materials Required

A.1.1 SM Broth

Ingredients:

0.05M Tris (FW = 121.4 = 6.057 g/L distilled H_2O)

NaCl 5.8 g/L

Supplements:

$MgSO_4$ 2.0 g/L

Gelatin 5mL of 2% w/v solution (0.2g/10mL)

Procedure:

1. Weigh Tris and NaCl and dissolve in distilled water. Adjust pH to 7.5 before autoclaving.
2. Add filter-sterilised (0.22µm) supplement of $MgSO_4$ (solution for adding 5mL/L of supplement $MgSO_4$ in 20mL distilled H_2O)
3. Add filter-sterilised (0.22µm) supplement of Gelatin

A.1.2 1.25mM $CaCl_2$ Solution

BDH $CaCl_2$ FW = 110.99g/M (100 ml = 0.1L)

110.99 g/M x 0.00125 M = 0.139 g/L

0.139 g /L x 0.1 = 0.0139g

0.0139g of $CaCl_2$ supplement dissolved in 100 mL of distilled water.

Autoclave: 121˚C, 15min.

All *Listeria* media was supplemented with 1.25mM $CaCl_2$ solution

A.1.3 10mM $MgSO_4$ Solution

$MgSO_4$ FW = 120.37g/M (100ml = 0.1L)

120.37 x 0.01 = 1.204 g/L

1.204 g/L x 0.1 = 0.1204g

1.204g of $MgSO_4$ supplement dissolved in 100mL of distilled water.

Autoclave at 121˚C, 15min.

All *E.coli* media was supplemented with 10mM $MgSO_4$ solution

A2 *E.coli* Media

A.2.1 LB Broth

25g Invitrogen LB Broth Base powder (Lennox L Broth Base)

Dissolved in 1L Distilled Water

Autoclave: 121˚C, 15 min

Final pH: 7.5 +/- 0.2

A.2.2 LB Agar

Invitrogen LB Broth Base 25g

Distilled water 1L

Merck Agar-Agar 15g

Autoclave: 121˚C, 15 min

Final pH: 7.5 +/- 0.2

A.2.3 LB Soft-Agar Overlay

Invitrogen LB Broth Base 25g

Distilled Water 1L

Merck Agar-Agar 6g

Dispense into glass test tubes in 3mL quantities with lid

Autoclave: 121˚C, 15 min

Final pH: 7.5 +/- 0.2

A.2.4 Double strength LB (dsLB)

Invitrogen LB Broth Base 50g

Distilled water 1L

Autoclave: 121˚C, 15 min

Final pH: 7.5+/- 0.2

A.2.6 BHI Broth

37g Bacto ™ BHI powder

Dissolved in 1L distilled water

Autoclave: 121˚C, 15 min

Final pH: 7.5 +/- 0.2

A.2.7 Components of MacConkey agar

50g MacConkey agar powder

1L Distilled water

Autoclaved: 121 °C, 15 min

Final pH: 7.5 +/- 0.2

A3 *Listeria* Media

A.3.1 TSB Broth

30g Tryptose Soy

1L Distilled water

Autoclaved: 121˚C, 15 min

Final pH: 7.5 +/- 0.2

A.3.2 TSB Agar

30g Tryptose Soy

1L Distilled Water

Merck Agar-Agar 15g

Autoclaved: 121˚C, 15 min

Final pH: 7.5 +/- 0.2

A.3.3 TSB Soft-Agar Overlay

Tryptose Soy 30g

Distilled Water 1L

Merck Agar-Agar 6g

Autoclave: 121˚C, 15 min

Final pH: 7.5 +/- 0.2

A.3.4 TSAYE Agar

Tryptose Soy 30g

Distilled water 1L

Merck Agar-Agar 12g

Yeast Extract: 6g

Autoclave: 121˚C, 15 min

Final pH: 7.5 +/- 0.2

A.3.5 TSAYE soft agar

Trypotose Soy 30g

Distilled water 1L

Merck Agar-Agar 4g

Yeast Extract: 6g

Autoclave: 121˚C, 15 min

Final pH: 7.5 +/- 0.2

A.3.6 Hsiang-Ning Tsai (HTM) minimal media for *Listeria* biofilm growth

Chemical	Stock	Volume	Quantity	Final volume	Sterilisation method
MOPS (pH 7.4)	1M	100ml	20.93g	10ml	Autoclave
KH_2PO_4	50mM	100ml	0.68g	9.64ml	Autoclave
Na_2HPO_4	100mM	100ml	1.4196g	11.55ml	Autoclave
$MgSO_4$	100mM	100ml	2.4647g	1.7ml	Autoclave
Glucose	500mM	100ml	0.901g	11ml	Filter
Thiamine	1mM	10ml	0.00337g	0.296ml	Filter
Riboflavin	1mM	10ml	0.00376g	0.133ml	Filter
Biotin	1mM	10ml	0.00244g	0.205ml	Filter
Lipoic acid	1mM	10ml	0.00206g	0.0024ml	Filter
Cystine	1mg/ml	10ml	0.01g	10ml	Filter
Methionine	1mg/ml	10ml	0.01g	10ml	Filter
$(NH4)_2SO_4$	10mg/ml	10ml	0.1g	6ml	Filter
H_2O				29.4736ml	
Total				**100ml**	